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Studies on the interaction of a checkpoint clamp with components of the base excision repair machinery

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1 Summary

1.1 English

The protein complex 'Rad9-Rad1-Hus1' ('9-1-1 complex') is a part of the cell machinery to detect DNA lesions. It has been shown to interact with DNA polymerase β (Pol β) and other proteins.

An interaction of the 9-1-1 complex with DNA ligase I could be shown by nickel-pulldown-assays with DNA ligase I and 9-1-1 complex. The interaction between Pol β and the 9-1-1 complex was investigated more closely by using three deletion constructs of Pol β. Immunoprecipitation assays, *in vitro* polymerase assays, *in vitro* polymerase stimulation assays and electrophoresis mobility shift assays indicated that the interaction of the 9-1-1 complex with Pol β is mediated by more than one interacting motif. Furthermore the importance of the lyase domain of Pol β for its affinity to DNA templates was revealed. Moreover, a sequence alignment with known interaction partners of the 9-1-1 complex resulted in the finding of 3 well conserved domains. Also the existence of these domains on apurinic endonuclease 1 showed another potential interaction partner. Structural analysis showed the putative binding motifs to be located on the outer surface of all the tested proteins.

In summary, the results obtained firstly support the hypothesis that the 9-1-1 complex acts as a recruiting platform for proteins involved in long patch base excision repair and secondly propose the existence of 3 interaction motifs forming an interaction site for the 9-1-1 complex with its partners.

1.2 German

Der Proteinkomplex 'Rad9-Rad1-Hus1' ('9-1-1 Komplex') ist ein Teil der Maschinerie, die in einer Zelle DNA Schäden entdeckt. Es ist bekannt, dass der 9-1-1 Komplex mit DNA polymerase β (Pol β) und anderen Reparaturproteinen interagiert. Eine Interaktion zwischen dem 9-1-1 Komplex und DNA ligase I konnte durch Nickel-pulldown-assays von gereinigtem Protein gezeigt werden. Des weiteren wurde die schon bekannte Interaktion vom 9-1-1 complex mit Pol β und drei deren Deletionsmutanten genauer untersucht. Die Resultate aus Immunopräzipitation Experimenten, *in vitro* polymerase Experimenten, *in vitro* polymerase stimulation Experimenten and "electrophoresis mobility shift" Experimenten zeigten, dass die Interaktion des 9-1-1 Komplexes mit Pol β durch mehr als eine Domäne vermittelt wird. Des weiteren konnte die Relevanz der Lyase-Domäne auf Pol β für die Bindung an die DNA ermittelt werden. Durch Sequenzvergleiche mit bekannten Interaktionspartnern des 9-1-1 Komplexes konnten 3 gut konservierte Domänen gefunden werden. Da diese Domänen auch auf der "apurinischen Endonuclease 1" existieren, stellt dieses Protein ein weiterer möglicher Interaktionspartner dar. Strukturanalysen der möglichen Bindungsdomänen zeigten, dass alle 3 davon auf der äusseren Oberfläche der getesteten Proteine zu finden sind.

Zusammenfassend unterstützen die Resultate erstens die Hypothese, dass der 9-1-1 Komplex als Rekrutierungsplattform für Proteine dient, die in der "Long patch" Basen Exzisionsreparatur involviert sind, und zweitens deuten sie auf die Existenz von 3 Bindungsdomänen hin, die die Bindung des 9-1-1 Komplexes mit seinen Interaktionspartnern formen.

2 Introduction

2.1 DNA stability, the cell cycle and DNA damage checkpoints

All the genetic information that enables a cell to develop, divide and react to its environment (shortly said to live) is stored on the DNA located in the cell nucleus. DNA, as a chemically highly reactive molecule, is a main target for various kinds of damaging agents. Risk factors that can cause severe damage to the DNA are not only derived from the cell environment, such as chemical and physical influences, but can also consist of intracellular metabolic intermediates such as oxygen, which can damage DNA. Finally, DNA polymerisation by DNA polymerases, which can accidentally misincorporate nucleotides during DNA replication [1] can lead to altered DNA. All these events can cause DNA damages that then may lead to incorrect DNA sequence or structure, representing a high risk situation to the cell.

To guarantee survival, a cell has to be able to replicate its DNA correctly or to repair eventual damage, in order to maintain the sequence and structure of this crucial molecule stable and active in physiological ranges over the entire lifespan. Over billions of years of evolution many different strategies have evolved in order to make a cell able to respond to those two tasks of replication and repair in the most efficient way possible. Only the most successful strategies have been conserved over this vast timespan by being constantly inherited to the subsequent cell generations. Those strategies mainly consist of enormous protein machineries coordinating the fundamental and complicated events that occur during a cell's life. It is striking, that, despite the evolutionary distance, the functions of those proteins and their underlying mechanisms are highly similar in all three kingdoms of life (prokaryotes, archaeas and eukaryotes) [2].

Furthermore, in an organism consisting of more than one cell, an additional task to be faced is the communication and coordination of the single cells, in order to create a symbiotically functioning complex in which the advantages for the whole complex are of higher interest than the fate of a single member. The loss of the ability of the cell to act as part of a major complex can lead to severe disorders and problems for the whole complex, sometimes resulting in pathological conditions such as cancer or hereditary diseases.

The cell life consists of a cycle, divided in 4 phases: the G1-phase (gap 1) as the phase of the cells normal activity state, followed by the S-phase (synthesis), representing the period in which the DNA is copied. Afterwards, cell enters the G2-phase (gap 2), a short phase to enable preparation and initiation of cell division, and finally the M-phase (mitosis), which can be subdivided again in different stages, where the cell divides itself in 2 identical daughter cells. As a fifth stage the G0-phase (gap 0), a steady equivalent of the G1-phase, represents the state of the cells that have undergone full differentiation and cannot divide further under normal circumstances.

The cell cycle progression is strongly regulated and surveyed by so called cell cycle checkpoints. By means of highly sophisticated pathways that can be activated at those checkpoints, the cell surveys the progression of every phase of the cell cycle, even under severe circumstances. The cellular response provoked by DNA damage consists of slowing down the cell cycle and stalling it completely at the G2/M transition (reviewed in [3]), to provide the necessary time to repair damage through multiple different DNA repair mechanisms (reviewed in [4]). Those mechanisms

include base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and double-strand break repair (DSBR). One of the main DNA polymerases involved in DNA repair is the DNA polymerase β, one of the main subjects in this thesis. In addition to this, among many other proteins important for DNA replication and repair, DNA ligase I is needed to ligate a freshly synthesized DNA strand to the pre-existing downstream strand, in order to guarantee a single, coherent new strand to be formed. DNA ligase I is the second protein which was looked more closely at in this thesis.

The DNA damage checkpoint (reviewed in [5] and [6]) is a signal transduction pathway, which, in case of DNA damage, either slows down the rate at which S phase proceeds or blocks the cell cycle progression completely. It acts as a surveillance mechanism that is not required for cell cycle events occurring in physiological conditions. However, after extensive damage of the DNA, the maintenance of the right order of events that take place in the cell cycle is critically threatened, as the cell tends to progress, even though not all the genome has been correctly replicated or errors remain unrepaired. This is the point where DNA damage checkpoints play a crucial role, in order to ensure that the order of the cell cycle events is maintained chronologically correct. Furthermore, the checkpoint pathway targets the induction of transcription of genes involved in repair processes as well as stabilisation of stalled replication forks and, in worst case, the induction of apoptosis, when integrity of the genome cannot be ensured anymore.

The checkpoint proteins can be divided into several groups.:

1. The phosphatindylinositol-3-kinase (PI3K)-like protein kinases which form a large group and can be further divided into two subgroups; one of them consists of large protein kinases named ATM (ataxia telangiectasia mutated) and the other of protein kinases called ATR (ataxia- and Rad-related). Both subgroups are thought to bind to damaged DNA and either one, the other or both are required for each of the DNA-damage-responsive checkpoints.

2. The PCNA-like group, which is a complex of Rad9, Rad1 and Hus1, forming a heterotrimer called the '9-1-1' complex, that can be loaded directly on the DNA at sites of DNA damage. This protein complex, representing the third protein-protagonist in this thesis work, was identified to be involved in the checkpoint cascade (al-Khodairy et al., 1994).

3. Further groups consist of the two serine/threonine (S/T)-kinases Chk1 and Chk2 and their adaptor proteins. Those are activated by ATM/ATR and phosphorylate targets of the checkpoint.

2.2 DNA ligase I

2.2.1 Structure and function of DNA ligase I

DNA ligases play a vital role in the important processes of DNA replication, recombination and repair. Their task is to catalyze the joining of breaks in the phosphodiester backbone of duplex DNA (either due to a cutting and polymerisation step or damage). Three related classes of ATP-dependent DNA ligases have been shown to exist in eukaryotic cells - DNA ligases I, III and IV (reviewed in [7] and [8]). One of those ATP-dependent ligases is DNA ligase I (molecular mass, 102 kDa),

which is required for the ligation of Okazaki fragments during lagging-strand DNA synthesis as well as for several DNA-repair pathways. In DNA synthesis, the main mediator between DNA ligase I and its DNA substrate is the sliding-clamp protein PCNA (proliferating cell nuclear antigen) [9], which is known to bind to DNA polymerase δ [10] and thus to be a DNA polymerase processivity factor, probably due to a role as coordinator of protein-protein interactions on DNA (reviewed in [11]).

DNA ligase I consists of 4 domains. Motif I includes the lysine residue as catalytic domain (CD) that is adenylated in the first step of the ligation reaction. Motif II is a non-catalytic domain (NCD) of unknown function. In addition to the CD and NCD domains, DNA ligase I also possesses an amino-terminal domain that includes a nuclear localization sequence (NLS) and, at the extreme amino terminus, a conserved PCNA-binding motif (PBM).

2.2.2 DNA ligase I in DNA repair processes

DNA ligase I is, in addition to its other functions, required for base excision repair (BER) (rewiewed in [7, 8]). This is a repair pathway in which a damaged base is removed from the DNA, leaving behind an abasic site. After incision of the strand (by APE1), removal of the 5'overhang (by Fen1) and polymerization of the nucleotides according to the template (by DNA polymerase β), the gap is finally sealed again by DNA ligase I. The first step in the ligation reaction catalysed by DNA ligase I is the formation of a covalent enzyme-AMP complex. ATP, needed as a cofactor in this reaction, is cleaved to pyrophosphate and AMP. Subsequently, the AMP is covalently joined to a highly conserved lysine residue in the active site of the ligase. The AMP residue, activated by this binding, is then transferred to the 5' phosphate of the nick. After this transfer, the nick is sealed by phosphodiester-bond formation and release of the AMP.

2.3 DNA polymerase β

2.3.1 Structure and function of DNA polymerase β

DNA polymerases (reviewed in [10]) are template-directed enzymes, catalyzing phosphoryl transfer reactions. Their role lies in synthesis of long polymers of nucleoside monophosphates, whose linear addition is given by the sequence of the complementary template DNA strand [12]. The structure of DNA polymerases has been optimized through evolution to suit the specialized tasks that each polymerase faces within its environment. Among the about 20 DNA polymerases known in an animal cell, the one that draws the attention in this thesis work is the DNA polymerase β.

DNA polymerase β is the smallest eukaryotic polymerase. It consists of a single 39 kDa polypeptide containing 335 amino acid residues (reviewed in [13]) and can be divided into two major domains. The small domain is the 8-kDa N-terminal domain, that performs the 5'-deoxyribose phosphatase activity (5'dRP-lyase activity) and binds single-stranded DNA. The larger 31-kDa domain performs the polymerisation

of desoxyribonucleotides to a primer template. This second, large domain can be divided further in 3 subdomains: the thumb, the palm and the finger subdomains. Those subdomains fold into a conformation resembling a human right hand composed of three distinct domains designated as palm, thumb, and fingers. The fingers are involved in the process of correctly positioning the template and the incoming complementary dNTP, whereas the thumb is important in DNA binding and processivity [13, 14]. The structure of DNA polymerase β has been solved by X-ray crystallography [15, 16].

2.3.2 DNA polymerase β in DNA repair processes

After DNA lesions have occurred, they are immediately recognized by DNA damage sensors and transducers [6]. The lesions are then processed by specialized multiprotein complexes, that lead to DNA repair via one of the repair pathways such as base excision repair (BER), nucleotide excision repair (NER), double-strand break repair (DSBR) or mismatch repair (MMR). An important component of these complexes are DNA polymerases that have the task to synthesize DNA during the DNA repair processes. In human cells, one member of the DNA polymerase X family, DNA polymerase β, has been shown to be the major repair polymerase and to be essential for the BER pathway. DNA polymerase β's preferential DNA substrate is a shortly gapped DNA substrate [10], on which it performs strand displacement synthesis [17]. It also possesses a 5'dRP lyase activity, which is essential for completion of BER [18-20]. Furthermore, it has been shown that DNA polymerase β is able to bypass *in vitro* several types of DNA lesions like apurinic (AP) sites, cisplatin (cis-Pt) adducts or cyclobutane pyrimidine dimer (CPD) lesions, though it displays a relatively low efficiency [21-24].

2.4 The Rad9-Rad1-Hus1 complex: Structure and function

To achieve activation of the cell cycle checkpoints in order to stop cell cycle progression due to DNA damage in eukaryotic cells, a wide variety of DNA damage sensors and transducers are needed. Some of those are ATM, ATR, ATRIP, Rad17, Rad9, Rad1 and Hus1 [6]. The three human proteins Rad9, Rad1 and Hus1 have been shown to form a heterotrimeric complex, called the 9-1-1 complex. The structure of this complex shares an obvious similarity with the homotrimeric clamp formed by proliferating cell nuclear antigen (PCNA) [25-28]. It was further shown, that the 9-1-1 complex can be loaded on the DNA by human Rad17-RF-C_{2-5}, thus functioning as clamp/clamp loader pair, as PCNA is loaded onto DNA by RF-C [29-32]. Further data suggests, that upon recognition of DNA lesions by Rad17-RF-C_{2-5}, the 9-1-1 complex is recruited to those sites, probably serving as a recruiting platform for the checkpoint effector kinases, such as Chk1 or Chk2 [33, 34]. The currently accepted model proposes, that the 9-1-1 complex is present where DNA repair is occurring, suggesting, that it may play a direct role in subsequent stages of repair processes. The fact that the 9-1-1 complex interacts physically and functionally with DNA polymerase β [35] and Fen1 [36], strengthens this model further specially considering that both proteins play vital roles in DNA repair processes.

3 Aim and Project description

Although both DNA repair and DNA damage checkpoint mechanisms are quite well known, the link between those two processes induced upon DNA damage remains unclear. However, some recent evidence indicated that the 9-1-1 complex acting as recruiting platform could play a direct role in subsequent stages of DNA repair. Studies with the aim to address the link between checkpoint proteins and DNA repair in terms of physical and functional interaction revealed that the 9-1-1 complex directly interacts with DNA polymerase β and stimulates its activity. Previous results from the Institute of Veterinary Biochemistry and Molecular Biology showed that (results published in [35]):

- DNA polymerase β interacts physically with the 9-1-1 complex and each of its subunits
- The 9-1-1 complex specifically stimulates DNA polymerase β by increasing its affinity for the primer/template
- The 9-1-1 complex stimulates the strand displacement synthesis of DNA polymerase β

Furthermore, DNA ligase I is involved in DNA repair in terms of being responsible for the last step of this process. It ligates the newly synthesised, repaired strand to the non-damaged remaining DNA in order to produce one single, coherent strand again [7, 8]. The interaction of the 9-1-1 complex with various enzymes involved in the DNA repair mechanism (Table 1) as well as the interaction with Fen 1 [36] and DNA polymerase β [35] has already been shown. Hence it was of great interest to study as well the possible interaction of the 9-1-1 complex with DNA ligase I, specially considering that 9-1-1 complex is proposed to be the recruiting platform for proteins involved in long patch base excision repair (LP-BER).

Table 1 was published in [36] and is shown here as slightly modified version.

LP-BER component	**Interacting clamp**	**Functional consequence**	**Reference**
DNA glycosylase	PCNA	Enhancement of base excision activity	[37, 38]
MutY DNA glycosylase	9-1-1 complex	Not determined	[39]
Apn2	PCNA	Stimulation of 3'→5' exonuclease and 3'-phosphodiesterase activities	[40]
HAP1	9-1-1 complex	Not tested	Not tested
DNA polymerase β	PCNA	No effect	[41]
DNA polymerase β	9-1-1 complex	Stimulation of polymerase and strand displacement activities	[35]
Fen1	PCNA	Stimulation of endo- and exo-nuclease activities	[42]
Fen1	9-1-1 complex	Stimulation of endo- and exo-nuclease activities	[43]
DNA ligase I	PCNA	Stimulation of ligase activity	[44]
DNA ligase I	9-1-1 complex	**Results presented in this work**	[45]

Table 1: Functional consequences of the interactions of the two clamps PCNA and 9-1-1 complex with components of the LP-BER machinery.

In a first part of this thesis the physical interaction of the 9-1-1 complex with the human DNA ligase I was investigated. This interaction study was done by use of Pulldown assays. The assay had first to be optimized for the DNA ligase I protein and then tested for possible interaction with the 9-1-1 complex. Those results were published in Smirnova et al. [45].

In the second part of this thesis the interaction site of DNA polymerase β with the 9-1-1 complex was mapped. This was done by Immunoprecipitation-assays, using three DNA polymerase β deletion constructs provided by Nazim El Andaloussi (described in [46]). The main part of this thesis work is based on those 3 deletion mutants. The mutants were deprived of special domains of wild type protein, called lyase, thumb or palm domain, respectively, offering the possibility to study the influence of those mutations on their activity, and draw possible conclusions about the functions of each of the domains. Furthermore, they enabled more specified interaction studies to be performed. These results aimed to contribute to better understanding of how the interaction between the 9-1-1 complex and DNA polymerase β occurs and how the 9-1-1 complex modulates the activity of DNA polymerase β. The first task for this part was to establish and optimize the Immunoprecipitation assay for DNA polymerase β full length and the His-tagged 9-1-1 complex. After this the deletion mutants were tested for their possible interaction with the His-9-1-1 complex in the same conditions, to determine the subdomain of DNA polymerase β involved in this interaction. Furthermore, *in vitro* DNA polymerase assays and electrophoresis mobility shift assays were carried out to investigate changes in DNA polymerisation activity or DNA binding ability of all the mutant proteins. The influence of the 9-1-1 complex on those activities was tested as well. As a last step some sequence and structure analysis was performed, resulting in the finding of three possible 9-1-1 complex interaction motifs on its binding partners.

4 Material and Methods

4.1 Buffers and Solutions

Buffer A
(Polβ purification)

27.7 mM KCl
80.2 mM K2HPO4
20 mM KH2PO4
350 mM NaCl
20% (v/v) glycerol
0.05% (v/v) NP-40
1 mM PMSF
1 mM DTT
1 μg/ml pepstatin
1 μg/ml bestatin
1 μg/ml leupeptin

Buffer B
(Polβ purification)

1 M KCl
80.2 mM K2HPO4
20 mM KH2PO4
350 mM NaCl
20% (v/v) glycerol
0.05% (v/v) NP-40
1 mM PMSF
1 mM DTT
1 μg/ml pepstatin
1 μg/ml bestatin
1 μg/ml leupeptin

Buffer H '0'
(His- & notag 9-1-1 purification)

25 mM HEPES pH 8.1
1 mM EDTA
8.7% (v/v) glycerol
0.01% (v/v) NP-40
10 mM β-mercaptoethanol
1 mM PMSF
20 μg/ml leupeptin

Buffer H '1'
(His- & notag 9-1-1 purification)

1 M NaCl
25 mM HEPES pH 8.1
1 mM EDTA
8.7% (v/v) glycerol
0.01% (v/v) NP-40
10 mM β-mercaptoethanol
1 mM PMSF
20 μg/ml leupeptin

Buffer H 'A'
(His-9-1-1 purification)

100 mM NaCl
25 mM HEPES pH 8.1
1 mM EDTA
8.7% (v/v) glycerol
0.01% (v/v) NP-40
10 mM β-mercaptoethanol
1 mM PMSF
20 μg/ml leupeptin

Buffer H 'B'
(His-9-1-1 purification)

300 mM imidazole
100 mM NaCl
25 mM HEPES pH 8.1
1 mM EDTA
8.7% (v/v) glycerol
0.01% (v/v) NP-40
10 mM β-mercaptoethanol
1 mM PMSF
20 μg/ml leupeptin

Coomassie destaining solution

10% (v/v) acetic acid
10% (v/v) isopropanol

Dialysis buffer
(Polβ purification)

20 mM Tris pH 7.5
20% (v/v) glycerol
1 mM β-Mercaptoethanol
100 mM NaCl

Elution buffer
(Polβ & His-9-1-1 purification)

2.7 mM KCl
80.2 mM K2HPO4
20 mM KH2PO4
10% (v/v) glycerol
500 mM Imidazole
0.05% (v/v) NP-40
1 mM PMSF
1 mM DTT
1 μg/ml pepstatin
1 μg/ml bestatin
1 μg/ml leupeptin

IP buffer

50 mM Tris pH 8
100 mM NaCl
0.05% (v/v) NP-40
1 mM PMSF
1 μg/ml leupeptin
1 μg/ml pepstatin
1 μg/ml bestatin

Coomassie staining solution

10% (v/v) acetic acid
0.25% (w/v) Coomassie Blue R250
40% (v/v) methanol

Dialysis buffer '9-1-1'

17.4 % (v/v) glycerol
100 mM NaCl
20 mM HEPES pH 8.1
1 mM EDTA
10 mM β-mercaptoethanol
1 mM PMSF
20 μg/ml leupeptin

EMSA-buffer

50 mM Tris–HCl pH 8.0
10 mM NaCl
5 mM EDTA
100 μg/ml bovine serum albumin
4% (w/v) Ficoll
50 fmol of labelled EMSA substrate

Extraction buffer
(Polβ purification)

2.7 mM KCl
80.2 mM K2HPO4
20 mM KH2PO4
350 mM NaCl
10% (v/v) glycerol
10 mM Imidazol
0.05% (v/v) NP-40
1 mM PMSF
1 μg/ml pepstatin
1 μg/ml bestatin
1 μg/ml leupeptin

IP washing buffer

50 mM Tris pH 8
100 mM NaCl
0.05% (v/v) NP-40
1 mM PMSF
1 µg/ml leupeptin
1 µg/ml pepstatin
1 µg/ml bestatin
5 mM imidazole

Lysis buffer
(9-1-1 purification)

0.5% (v/v) NP-40
50 mM Tris pH 8
150 mM NaCl
1 mM EDTA
8.7 % (v/v) glycerol
10 mM β-mercaptoethanol
1 mM PMSF
20 µg/ml leupeptin

Stop buffer (2 x)

96% (v/v) formamide
20 mM EDTA
bromophenol blue
xylene cyanol

Pulldown washing buffer

50 mM Tris pH 8
100 mM NaCl
0.05% (v/v) NP-40
1 mM PMSF
1 µg/ml leupeptin
1 µg/ml pepstatin
1 µg/ml bestatin
5 mM imidazole

Lämmli buffer (2 x)

500 mM Tris pH 7.5
4% (w/v) SDS
20% (v/v) glycerol
40 mM DTT
0.02% (w/v) bromophenol blue
pH 7.5

Pulldown buffer

50 mM Tris pH 8
100 mM NaCl
0.05% (v/v) NP-40
1 mM PMSF
1 µg/ml leupeptin
1 µg/ml pepstatin
1 µg/ml bestatin

TBE buffer (10 x)

90 mM Tris
90 mM boric acid
20 mM EDTA

TBS

10 mM Tris pH 8
50 mM NaCl

TBS-T

10 mM Tris pH 8
50 mM NaCl
0.1% (v/v) Tween

Running buffer (SDS-PAGE)

1.5 M Tris pH 8
0.4% (w/v) SDS

Washing buffer **(Polβ & His-9-1-1 purification)**
2.7 mM KCl 80.2 mM K2HPO4 20 mM KH2PO4 10% (v/v) glycerol 10 mM Imidazole 0.05% (v/v) NP-40 1 mM PMSF 1 mM DTT 1 μg/ml pepstatin 1 μg/ml bestatin 1 μg/ml leupeptin

Transfer buffer
25 mM Tris-Base 192 mM glycine 20% (v/v) methanol

5 x annealing buffer
100 mM Tris pH 7.4 750 mM NaCl

4.2 Proteins

4.2.1 DNA polymerase β wild type

The DNA polymerase β wild type protein was cloned, transfected, expressed and purified by Nazim El Andaloussi [46], according to his protocol.

4.2.2 DNA polymerase β delta lyase

The DNA polymerase β delta lyase protein was cloned, transfected, expressed and purified by Nazim El Andaloussi [46].

4.2.3 DNA polymerase β delta thumb

The DNA polymerase β delta thumb protein was cloned by Nazim El Andaloussi [46].

The purification of the protein was then performed as following. The cell pellet from 1 l culture was resuspended in 30 ml extraction buffer and then lysed by compressing them twice in the French Press. The lysate was centrifuged 30 min at 20'000 rpm at +4°C and the supernatant was loaded on a Ni-NTA HiTrap column previously equilibrated with degassed washing buffer and degassed elution buffer. The flow-through was collected and reloaded later on. After washing the column with 5 column volumes (column volume = 1 ml) of washing buffer, the elution from the Ni-NTA HiTrap column was performed by using a continuous gradient from the washing buffer to the elution buffer (10 – 500 mM imidazole) in 10 column volumes, collecting fractions of 250 μl volume. Then the column was washed once again with 5 column volumes of elution buffer to elute eventually retained protein from the

column. After this first elution, the flow-through was loaded and eluted according to the same procedure.
To determine the fractions to be pooled and loaded on the next column, 10 µl of every third fraction was loaded on a 12 % SDS-PAGE gel [Acrylamide/Bis, solution (37.5:1), 40% (w/v) (Serva)]. The gel was stained then by Coomassie staining solution for 45 min and destained by washing 3 times 30 minutes with Destaining solution. The same was done for the flow-through-elution pools. The eluted samples were then pooled according to the Coomassie-gel and the new pools were diluted 1:10 in Buffer A to reduce the imidazole concentration in the solution. The first elution pool was then loaded on a Hi-trap SP column, previously equilibrated with degassed buffer A and degassed buffer B, and eluted using the same elution profile as for the previous column (0 – 1 M NaCl). The flowthrough was collected, reloaded together with the second elution pool from the first column and eluted after the same scheme. A 12 % SDS-PAGE gel was loaded with various fractions from the elution and analysed by Coomassie staining and Destaining as described above. The samples containing the protein were pooled into 3 different pools (High, Low1, Low2) and dialysed in dialysis cassettes (Slide-A-lyzer® 10K, Pierce) against 3 x 700 ml of Dialysis buffer. They were aliquoted by making "Popcorn" in liquid nitrogen and stored in liquid nitrogen.

4.2.4 DNA polymerase β delta palm thumb

The DNA polymerase β delta palm delta thumb protein was cloned, transfected, expressed and purified by Nazim El Andaloussi [46].

4.2.5 The untagged '9-1-1' complex

The purification of the human Rad9-Rad1-Hus1 complex ('9-1-1 complex') was done following a slightly modified protocol from Shiomi et al. [26]. The human 9-1-1 complex in its native form was expressed by co-expression of baculoviruses containing human Rad9, Hus1 and Rad1 cDNA sequences (provided by T. Tsurimoto, Japan) in insect SF-21 cells. Fractions containing equivalent amounts of these three proteins from insect cell lysates were obtained. The purification was performed by use of 3 different columns. The first column consisted of DEAE (=Diethylaminoethyl) Sepharose ® (Amersham Pharmacia Biotech), as second column a Hitrap Q was used and the third was a Hitrap SP. 72 h after infection, 1 x 10^8 infected SF-21 insect cells were lysed in 10 ml lysis buffer at 0°C for 30 min. The lysate was then centrifuged at 20'000 rpm for 2 h at 4°C (using a SS34 Rotor, RC5C, Sorvall © Instruments) and the supernatent was loaded on the DEAE Sepharose column, previously equilibrated with degassed buffer H '0' and degassed buffer H '1'. The protein was eluted from the column by 40% buffer H '1' (= 400 mM NaCl) and collected according to rising optical density (O.D = 280 nM). The DEAE-elution was diluted 1:4 in buffer H '0' and centrifuged 10 min at +4°C at 10'000 rpm, and the supernatant was loaded on the Hitrap Q (column volume = 1 ml) column previously equilibrated as described above for the DEAE Sepharose column. The column was washed with 5 column volumes of 10% buffer H '1' (= 100 mM NaCl). After this, the sample was eluted by a gradient

elution from 100 mM NaCl to 800 mM NaCl, followed by a step to 1 M NaCl and additional 5 column volumes of 1 M NaCl. Fractions of 500 µl were collected. To investigate which fractions to load on the next column, a 10% SDS-PAGE gel was run and a Coomassie staining and a western blot was performed with the corresponding antibodies for Hus1 (see 4.3.3), Rad1 (see 4.3.4) and Rad9 (see 4.3.2). The pool was diluted again in buffer H '0' to achieve a final NaCl concentration of about 50 mM. The pool was then loaded again on a Hitrap SP HP column. The elution was done by first washing the column with 5 ml (= 5 column volumes) of 5% buffer H '1' (50 mM NaCl) and then performing a step elution with 10 column volumes of 100 % buffer H '1' (1 M NaCl). A 10% SDS-PAGE gel was run and a coomassie staining was performed to determine which fractions to pool as the final protein sample. The pools were then dialysed in 3 times 500 ml of dialysis buffer '9-1-1', aliquoted and kept on –80°C.

4.2.6 The His-tagged 9-1-1 complex

The purification of the human Rad9-Rad1-(His)Hus1 complex ('9-1-1 complex', His-tagged) was optimised in our lab. The human 9-1-1 complex in a His-tagged form was expressed by co-expression of baculoviruses containing human Rad9, His-Hus1 and Rad1 cDNA sequences in insect SF-21 cells. Fractions containing equivalent amounts of these three proteins from insect cell lysates were obtained. 72 h after infection, 1 x 10^8 infected SF-21 insect cells were lysed in 10 ml lysis buffer at 0°C for 30 min. The lysate was then centrifuged at 20'000 rpm for 2 h at 4°C (using a SS34 Rotor, RC5C, Sorvall © Instruments) and the supernatant was loaded on a Ni-NTA HiTrap column (Amersham Biosciences), previously equilibrated with degassed buffer H 'A' and degassed buffer H 'B'. After washing the column with 5 column volumes (column volume = 1 ml) of buffer H 'A', the elution from the column was performed by using a continuous gradient from buffer H 'A' to buffer H 'B' (0 – 300 mM imidazole) in 10 column volumes. To clean the column of eventually still retained protein, the imidazole concentration was kept high for 5 additional column volumes. To investigate which fractions to load on the next column, a 10% SDS-PAGE gel was run and a Coomassie staining and a western blot were performed with the corresponding antibodies for Hus-1 (see 4.3.3), Rad1 (see 4.3.4) and Rad9 (see 4.3.2). The new pool was diluted 1:4 in buffer H '0' and centrifuged 10 min at +4°C at 10'000 rpm, and the supernatent was loaded on the Hitrap Q (column volume = 1 ml) column previously equilibrated with buffers H '0' and'1'. The column was washed with 5 column volumes of 10% buffer H '1' (= 100 mM NaCl). After this, the sample was eluted by a gradient elution from 100 mM NaCl to 800 mM NaCl, followed by a step to 1 M NaCl and additional 5 column volumes of 1 M NaCl. Again a 10% SDS-PAGE gel was run and a coomassie staining was done to determine which fractions to pool as the final protein sample. The pools were then dialysed in 3 times 500 ml of dialysis buffer '9-1-1', aliquoted and kept on –80°C.

4.2.7 Bovine serum albumin

The BSA used was purchased from New England Biolabs.

4.3 Antibodies

4.3.1 Anti-DNA polymerase β antibody

The mouse DNA polymerase β specific antibody 18S was purchased from Neo Markers. It recognizes the amino acids 140-154 which are present in the wild type as well as in all the three mutants (DNA polymerase β delta lyase, DNA polymerase β delta thumb, DNA polymerase β delta palm delta thumb). Therefore all the Western blots could be performed using the same antibody.

4.3.2 Anti-Rad9 antibody

The hRad9 cDNA was fused to the GST of the vector pGEX 4T-3 (Pharmacia). The hRad9 protein was mainly insoluble. The protein was purified according to the manufacturer's instructions. Antibodies against the recombinant protein were raised in rabbit by standard procedures [47]. This antibody was a gift from R. Freire (Teneriffe, Spain).

4.3.3 Anti-Hus1 antibody

The hHus1 cDNA (amino acid 1–134) was cloned and fused to the hexahistidine tag of the vector pQE-30 (Qiagen). The hHus1 protein was mainly insoluble. It was purified according to the manufacturer's instructions. Antibodies against the recombinant protein were raised in rabbit by standard procedures [47]. This antibody was a gift from R. Freire (Teneriffe, Spain).

4.3.4 Anti-Rad1 antibody

The goat anti-Rad1 antibody (N-18) was purchased from Santa Cruz biotechnology.

4.3.5 Secondary antibodies

Anti-Mouse and Anti-Rabbit IgGs were obtained from Amersham Biosciences.
HRP-conjugated donkey anti-goat antibody was from Santa Cruz Biotechnology.

4.4 DNA substrates, primers and oligonucleotides

4.4.1 17/73 mer template for DNA polymerase assays

The 17 and 73 mer (primer/template) were chemically synthesized and purified on denaturing polyacrylamide gels in the laboratory of Giuseppe Villani (Toulouse, France). The sequences are the following:

d73mer:

5'-GATCGGGAGGGTAGGAATATTGAGGATGAAAGGGTTGAGTTGAGTGGA GATAGTGGAGGGTAGTATGGTGGATA-3'

d17mer:

5'-TATCCACCATACTACCC-3'

4.4.2 Electrophoresis mobility shift assay (EMSA) oligonucleotide substrate

The template consists of a 100 mer one nucleotide gapped substrate of d100 mer, d42 mer (upstream oligonucleotide) and d57 mer (downstream oligonucleotide).

4.5 Nickel-Pulldown of untagged 9-1-1 complex via His-DNA ligase I

For detailed Material and Methods, see pulldown experiments in the published results in Smirnova et al., 2005 [45], enclosed in this thesis.

4.6 Immunoprecipitation assay of His DNA polymerase β wild type, His DNA polymerase β delta lyase, His DNA polymerase β delta thumb and His DNA polymerase β delta palm delta thumb by his-9-1-1 complex

3.6 μg (= 154 pmol) of purified His-tagged DNA polymerase β wild type, 4.23 μg (=154 pmol) of DNA polymerase β delta lyase, 4.65 μg (=154 pmol) of DNA polymerase β delta thumb or 2.74 μg (= 154 pmol) of DNA polymerase β delta palm delta thumb, respectively, were preincubated with 3.6 μg of purified His-9-1-1 complex in a final volume of 30 μl in IP-buffer on a roller shaker at +4°C for 2 hours. 2 μl of anti-Rad9 antibody was added and incubated for 1 h at +4°C. 60 mg of protein A sepharose were coated by incubation overnight in 1 ml of IP buffer containing 1 mg boiled BSA. After centrifuging 2 min at 1500 rpm at +4°C (centrifuge 5417R, Eppendorf), the supernatent was discarded and 1 ml of washing buffer was added. This washing procedure was repeated 3 times, every time with 10 min of incubation time on the roller shaker at +4°C. Then the beads were resuspended in 500 μl IP

buffer. 40 µl of this equilibrated protein A sepharose beads-slurry was then added to the reaction and the reaction was incubated for 1 more hour at +4°C. After washing again three times in washing buffer as described above, the supernatant was removed and 20 µl Lämmli buffer was added. The co-precipitated proteins were resolved on a 10% SDS-PAGE gel for 9-1-1 complex or a 12% SDS-PAGE gel for DNA polymerase β, respectively, and analyzed by western blot using the corresponding antibodies.

For negative control reactions, anti-Rad9 antibodies were added to a mix with DNA polymerase β only, leaving away the 9-1-1 complex. This showed us if a unspecific binding of DNA polymerase β to protein A sepharose beads coated with anti-Rad9 antibody occurred or not.

4.7 Western blot

Proteins were separated on a SDS-PAGE [Acrylamide/Bis, solution (37.5:1), 40% (w/v) (Serva)] and electroblotted during 1.5 h at 120 V (BioRad Western Blot apparatus) onto a PVDF Membrane (Immobilon) in transfer buffer at + 4 °C. After blocking the membranes for 45 min in TBS-T with 2.5% powdered Milk, the membranes were incubated with the appropriate antibodies, diluted in TBS-T with 2.5% powdered Milk, for 4 hours at room temperature. Then the membranes were washed 3 times 10 minutes in TBS-T and incubated with the corresponding secondary antibodies, diluted in TBS-T with 2.5% powdered Milk, for 1 hour. This was followed by washing again the membranes 3 times 10 min in TBS-T and additionally 2 times 5 min in TBS. The antibodies bound to the membranes were then detected by Uptilight HRP Blot Chemoluminescent Substrate (Uptima) and exposure to a X-ray film (Contatyp, Typon Imaging AG)

4.8 Preparation of radiolabeled template

To prepare the 17/73 mer primer template, the 17 mer primer was labeled at its 5' end by mixing the following reagents in a reaction mix to a final volume of 20 µl:

3.6 mM 17 mer
20 µCi [γ-32^P]dATP (Amersham Biosciences)
2µl 10 x PNK buffer (New England Biolabs)
0.6µl T4 polynucleotide kinase (New England Biolabs)

The mix was incubated for 40 min at 37°C and then heat inactivated for 10 min at 80°C and centrifuged through a G-25 (Microspin ™) column at 720 x g for 2 min to remove the free ATP. The reaction was then added to the annealing mix containing following reagents to a final volume of 36 µl:

3.6 mM 73 mer
7.2 µl 5 x annealing buffer

This mix was then heated at 95°C for 10 min and cooled down overnight slowly

to room temperature to allow the primer to anneal to the template.

The 1 nucleotide gapped substrate was prepared by 5' labeling of the upstream 42 mer oligonucleotide, followed by annealing of this oligonucleotide with equimolar amounts of the 100 mer template and the 57 mer downstream oligonucleotide, according to the protocol described above.

4.9 DNA polymerase assay

The DNA polymerase reaction was performed in a final volume of 10 µl containing: 50 mM Tris pH 7.5, 2.5 mg/ml BSA, 10 mM DTT, 50 µM 5' labelled 17*/73 mer, 1 mM $MnCl_2$ and 100 µM dNTP's (Roche). The reaction was started by adding 9 µl of the reaction mix to DNA polymerase β dilutions done in 1 µl and then incubated at 37 °C for 15 min. Finally, 10 µl of 2 x Stop buffer was added to each reaction tube and the reaction was put on ice. The samples were heated for 3 min at 95°C and loaded on a 10% denaturing polyacrylamide (Acrylamide/Bis-acrylamide, 19:1 mixture, 40% (w/v) (Qbiogene)) sequencing gel containing 7 M urea. The result was visualised by exposing the gel to a X-ray film (Contatyp, Typon Imaging AG) overnight at –80°C, using a radioactivity-sensitive screen (Okamoto).

4.10 DNA polymerase β stimulation assay by 9-1-1 complex

His-9-1-1 dilutions (amounts as indicated) were done in 1 µl and were complemented with BSA, in order to maintain a constant total protein amount in the dilutions. 2 µl of 1:1 His-9-1-1/BSA dilutions was preincubated in 6.5 µl of preincubation mix consisting of 50 mM Tris pH 7.5, 2.5 mg/ml BSA, 10 mM DTT, 50 µM 5' labelled 17*/73 mer and 135 nM DNA polymerase β wild type for 15 min on ice. Then 1.5 µl of starting-mix [100 µM dNTP's (Roche) and 1 mM $MnCl_2$, final concentrations] was added and the reaction incubated for 15 additional minutes at 37°C. The reaction was stopped by adding 10 µl of 2 x Stop buffer and putting the samples on ice. The samples were heated for 3 min at 95°C and then loaded on a 10% denaturing polyacrylamide (Acrylamide/Bis-acrylamide, 19:1 mixture, 40% (w/v) (Qbiogene)) sequencing gel containing 7 M urea. The result was visualised by exposing the gel to a X-ray film (Contatyp, Typon Imaging AG) overnight at –80°C, using a radioactivity-sensitive screen (Okamoto).

4.11 Electrophoresis mobility shift assay (EMSA) for DNA polymerase β and its deletion mutants

Binding reactions with 50 fmol of 1 nt gap DNA substrate (see Figure 5, panel A) were carried out in a total volume of 20 µl in EMSA-buffer using the indicated amounts of DNA polymerase β or its mutants (Figure 5, panels B-E). After incubation at room temperature for 30 minutes, reactions were loaded on 6% polyacrylamide gels containing 0.5x TBE and run first at 50 V for 45 minutes and then at 100 V for 3

hours. The bands were visualized by autoradiography.

4.12 Alignment-search

The search for possible alignments was done using the ClustalW program from the European Bioinformatics Institute.

http://www.ebi.ac.uk/clustalw/#

Manufacturers description: “Clustal W is a general purpose multiple sequence alignment program for DNA or proteins. It produces biologically meaningful multiple sequence alignments of divergent sequences. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen. Evolutionary relationships can be seen via viewing Cladograms or Phylograms.” The sequences of the proteins used for alignment were obtained from the NCBI-website using the entrez-protein database.

4.13 Structural models of DNA polymerase β, DNA ligase I and apurinic endunuclease I

The structural models were visualized using the program ‘RasMol’, version 2.7.2.1.1 [48]. The models to use in this program can be found in the “MMDB Structure Summary” [49].

Model of DNA polymerase β: 8ICK; Dna Polymerase Beta (Pol B) (E.C.2.7.7.7) Complexed With Seven Base Pairs Of Dna; Soaked In The Presence Of Datp (1 Millimolar), Mgcl2 (5 Millimolar), And Mncl2 (5 Millimolar)

Model of DNA ligase I: 1X9N; Crystal Structure Of Human DNA Ligase I Bound To 5'- Adenylated, Nicked DNA

Model of apurinic endonuclease 1 (APE1): 1BIX; The Crystal Structure Of The Human DNA Repair Endonuclease Hap1 Suggests The Recognition Of Extra-Helical Deoxyribose At DNA Abasic Sites

5 Results

5.1 Human DNA ligase I interacts with the 9-1-1 complex

To test the possible interaction between human DNA ligase I and the 9-1-1 complex, a pulldown assay was performed. After some optimization steps, including testing different NaCl and NP-40 conditions, 100 mM NaCl and 0.1 % of NP-40 showed to be optimal. As an additional control the 'non-binder' GST was included. The results of this pulldown assay can be viewed in Smirnova et al., 2005, Figure 1, enclosed in this thesis under point 8. In the pulldown assay analysis a specific protein-protein interaction between the 9-1-1 complex and DNA ligase I under the given conditions was observed. The 9-1-1 complex was present as a complex, which was verified by western blot analysis against Rad9, Rad1 and Hus1. Furthermore, GST did not bind to DNA ligase I under these conditions, confirming the specificity of this interaction. These data contribute to additionally strengthening the model, that the 9-1-1 complex might act as a recruiting platform for proteins involved in LP-BER, similarly to PCNA in the replication machinery.

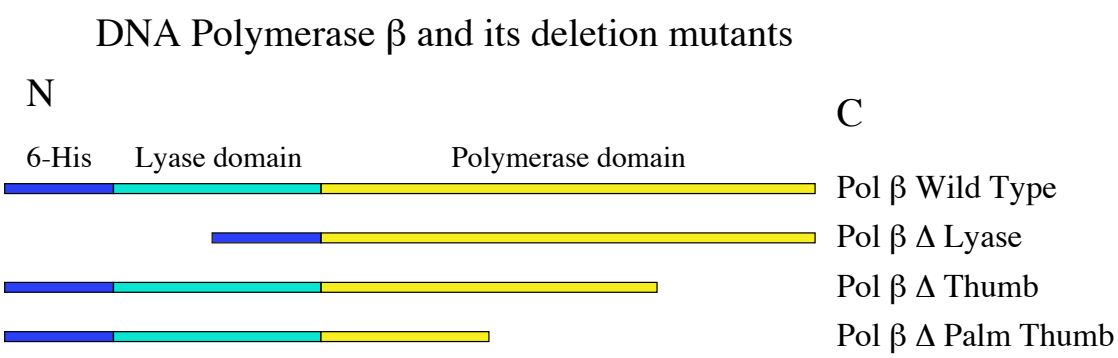
5.2 Characterization of DNA polymerase β deletion mutants

The DNA polymerase β and its deletion mutants are schematically presented in Figure 1, panel A. The proteins all contain a 6-histidine residue N-terminal tag. The DNA polymerase β wild type protein consists of 335 amino acids, of which the sequence is displayed in Figure 1, panel B. Its molecular weight is 36 kDa. The DNA polymerase β delta lyase mutant lacks the N-terminal part of the wild type DNA polymerase β, known as 'lyase-domain' (Figure 1, panels A and B). The deletion consists of the amino acids 1-101. Thus, the delta lyase protein is composed of the remaining amino acids 102-335, with a molecular weight of 27 kDa. The DNA polymerase β delta thumb mutant consists of the amino acids 1-262 of the wild type DNA polymerase β (Figure 1, panels A and B). It has been deleted of its C-terminal part of the amino acids 263-335, also referred to as the thumb domain. The molecular weight of this mutant is 29.7 kDa. The DNA polymerase β delta palm thumb mutant is deprived of the amino acids 156-335, therefore lacking a big part of the C-terminal part of the wild type protein (Figure 1, panels A and B). The deleted part is referred to as the thumb and the palm domain of the wild type protein. This mutant has a molecular weight of 17.5 kDa.

In order to check the DNA polymerase β deletion mutants for correct size and protein concentration and to rule out eventual contamination of the batches by other proteins, 12% SDS-PAGE was loaded with 2 μg of each protein, previously resuspended in Lämmli buffer and boiled for 5 minutes at 95°C. The gel was stained with Coomassie blue and destained. The result (Figure 2) showed, that the mutant batches are clearly free of DNA polymerase β wild type contamination and are of a quite high purity. The mutant proteins run in the gels as expected according to their molecular weight, which was important to know for the immunoprecipitation –

western blots performed later on. DNA polymerase β wild type (polβ WT) showed some degradation, probably due to long storage of this aliquot on –20°C.

A



B

Protein sequences of Polβ and its deletion mutants

```
  1 MSKRKAPQET LNGGITDMLT ELANFEKNVS QAIHKYNAYR KAASVIAKYP

 51 HKIKSGAEAK KLPGVGTKIA EKIDEFLATG KLRKLEKIRQ DDTSSSINFL
      Δ Lyase
101 TRVSGIGPSA ARKFVDEGIK TLEDLRKNED KLNHHQRIGL KYFGDFEKRI

151 PREEMLQMQD IVLNEVKKVD SEYIATVCGS FRRGAESSGD MDVLLTHPSF
Δ Palm Thumb
201 TSESTKQPKL LHQVVEQLQK VHFITDTLSK GETKFMGVCQ LPSKNDEKEY

251 PHRRIDIRLI PKDQYYCGVL YFTGSDIFNK NMRAHALEKG FTINEYTIRP
        Δ Thumb
301 LGVTGVAGEP LPVDSEKDIF DYIQWKYREP KDRSE
```

Figure 1: Schematic presentation of DNA polymerase β and its deletion mutants used in this thesis.
*(**A**) Scheme of DNA polymerase β wild type and its deletion mutants. The proteins all contain a 6-His-tag on the N-terminal part. The lyase domain is present in all of the proteins except in the delta lyase mutant. The full polymerase domain is present in the wild type and the delta lyase construct. The delta thumb construct has a polymerase domain lacking the last 73 amino acids on the C-terminal end. The delta palm thumb construct lacks 180 last C-terminal amino acids. (**B**) Sequence of the DNA polymerase β wild type. The small straight arrows indicate the amino acids, which surround the truncation sites. The bent arrows show the mutant protein remaining, labelled with the names of the mutants.*

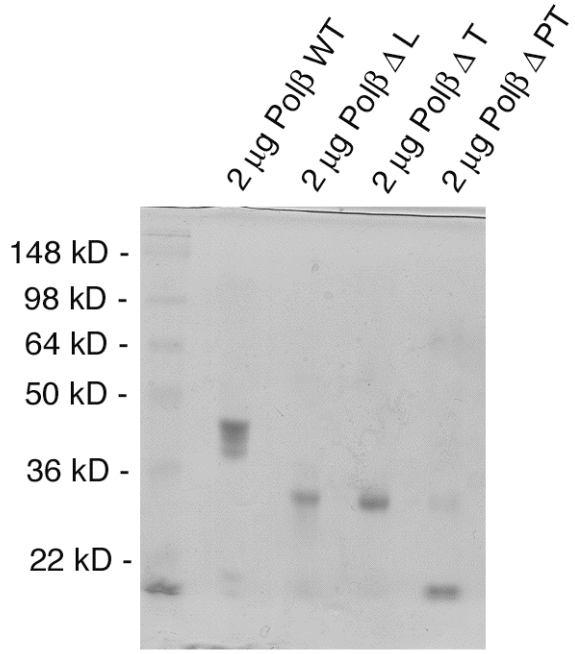


Figure 2: Coomassie staining of DNA polymerase β wild type and its deletion constructs. 2 µg of each protein was loaded onto an SDS-PAGE gel and separated as outlined in Materials and Methods. Polβ WT = DNA polymerase β wild type, Polβ Δ L = DNA polymerase β delta lyase, Polβ Δ T = DNA polynerase β delta thumb, Polβ Δ PT = DNA polymerase β delta palm thumb.

5.3 DNA polymerase β wild type interacts with the 9-1-1 complex

In order to confirm the interaction studies between 9-1-1 complex and DNA polymerase β wild type, and to be able to test the deletion mutants of DNA polymerase β under optimal conditions, first an Immunoprecipitation assay had to be established and optimized. For this, a NaCl concentration of 100 mM and an amount 0.05% (v/v) NP-40 was chosen to be the starting conditions, which had proved to be suitable to show this interaction experiments and to reflect also physiological conditions.

First, immunoprecipitation of a DNA polymerase β amount of 6 µg and a 9-1-1 complex amount of 3.6 µg was chosen, using either 2.5 µl or 1 µl of anti-Rad9 antibody (Figure 1) or 2.5 µl anti-Rad1 antibody (data not shown), to see which amounts of antibody and which one of the antibodies worked best. The negative controls were either the 9-1-1 complex and DNA polymerase β with preimmuneserum (the same amount as anti-Rad9 antibody) or anti-Rad9 antibody and DNA polymerase β in the absence of the 9-1-1 complex. From previous work done in this lab, it was expected, that the anti-Rad1 antibody was the most efficient for immunoprecipitation studies. However, the anti-Rad9 antibody, in addition to recognising specifically Rad9, recognises also the heavy chain of antibodies. Unfortunately Rad9 and heavy chains are both running at the same size, which makes it impossible to visualise Rad9

by a western blot against Rad9 protein after immunoprecipitation. For this reason DNA polymerase β was preferably immunoprecipitated by the anti-Rad9 antibody, in order to be able to show that all the three proteins of the 9-1-1 complex were present. Anti-Hus1 had been shown to be the least effective antibody for those interaction studies. For this and the same reason as for anti-Rad1 antibody, the antibody to be preferred was in any case the anti-Rad9. The result (Figure 3) showed, that the anti-Rad9 antibody could well be used in our studies, even with only 1 µl per assay. Under both conditions DNA polymerase β was co-immunoprecipitated by the 9-1-1 complex bound to protein-A sepharose beads via the anti-Rad9 antibody, but did clearly not bind to the beads either via the 9-1-1 complex and preimmuneserum or via anti-Rad9 antibody in the absence of the 9-1-1 complex (Figure 3, panel A). Furthermore, the best and most important negative control proved to be the one with anti-Rad9 antibody, no 9-1-1 complex and DNA polymerase β, as this could show clearly, if any of the DNA polymerase β bound to the beads unspecifically via affinity to the anti-Rad9 antibody. This control was then chosen for all subsequent experiments. In addition panel B and C in Figure 2 show that the entire 9-1-1 complex was precipitated by the anti-Rad9 antibody, since Rad1 and Hus1 could be detected in the immunoprecipitation as well.

It was furthermore of interest to test a reduced amount of DNA polymerase β, since the system should not be oversaturated and the aim was to use equimolar amounts of DNA polymerase β deletion mutants later. To test this, the amount of DNA polymerase β used was reduced to 3.6 µg in parallel with the amount of anti-Rad9 antibody that was lowered to 2 µl. When these lower amounts of DNA polymerase β were used, the DNA polymerase β could still be immunoprecipitated by the 9-1-1 complex bound to the protein-A sepharose beads, but not via unspecific binding through anti-Rad9 antibody (Figure 4, panel A).

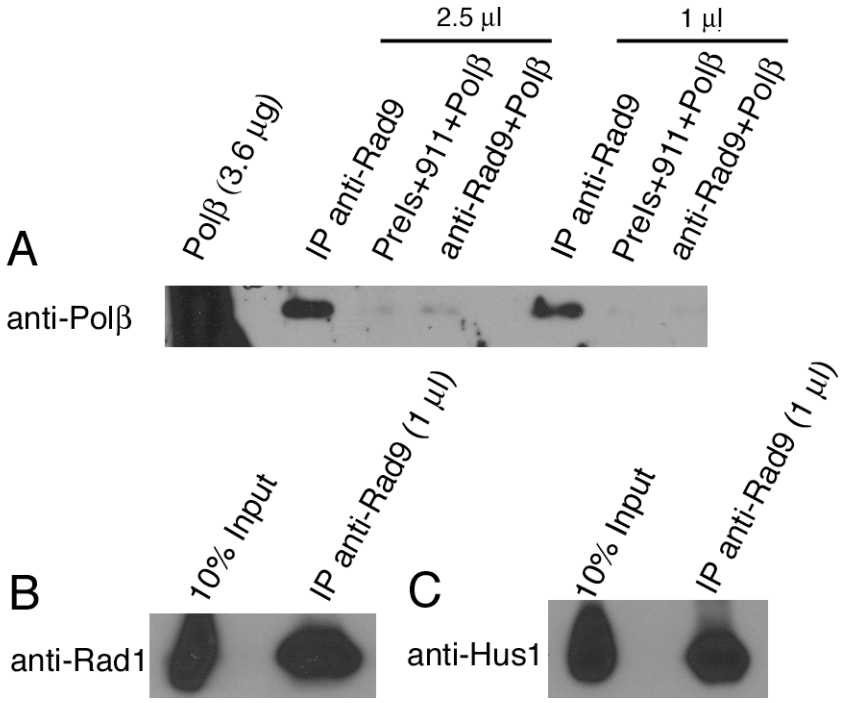


*Figure 3: Optimization of the immunoprecipitation of the His-9-1-1 complex and DNA polymerase β wild type. (**A**) Protein-A sepharose immunoprecipitations were performed in the presence of either 2.5 µl or 1 µl of anti-Rad9 antibody and 3.6 µg 9-1-1 complex and 6 µg DNA polymerase β. Negative controls were either 2.5 µl or 1 µl preimmuneserum (PreIs) and 3.6 µg 9-1-1 complex and 6 µ DNA polymerase β or 2.5 µl or 1 µl of anti-Rad9 antibody with 3.6 µg DNA polymerase β only. (**B**) The presence of Rad1 protein in the 9-1-1 complex was tested by analyzing a part of the immunoprecipitation with anti-Rad1 antibody. (**C**) The presence of Hus1 protein in the 9-1-1 complex was tested by analyzing a part of immunoprecipitation with anti-Hus1 antibody. The presence of co-precipitated proteins was determined by SDS-PAGE followed by Western blot analysis against the indicated proteins.*

5.4 DNA polymerase β likely interacts with the 9-1-1 complex via its palm domain

To investigate, in which subdomain the 9-1-1 complex binds to pol β, the immunoprecipitation assay described was performed with the 3 deletion mutants, using the same conditions as for the wild type form of DNA polymerase β. The mutants were used in equimolar amounts as the DNA polymerase β wild type, of which 3.6 µg was used. The results showed that

I the DNA polymerase β delta lyase mutant interacts with the 9-1-1 complex (Figure 4, panel B);

II the DNA polymerase β delta thumb mutant interacts with the 9-1-1 complex (Figure 4, panel C);

III the DNA polymerase β delta palm delta thumb mutant does not show any interaction with the 9-1-1 complex (Figure 4, panel D)

IV the 9-1-1 complex is always present as a complex, since Rad1 (Figure 4, panel E) and Hus1 (Figure 4, panel F) were present as co-immunoprecipitated proteins in all of the 4 different immunoprecipitation assays.

These results suggested, that the domain of pol β which mediates its interaction with 9-1-1 complex is most likely the palm domain, as a loss of specific interaction between the two proteins could be observed when this part of DNA polymerase β was deleted.

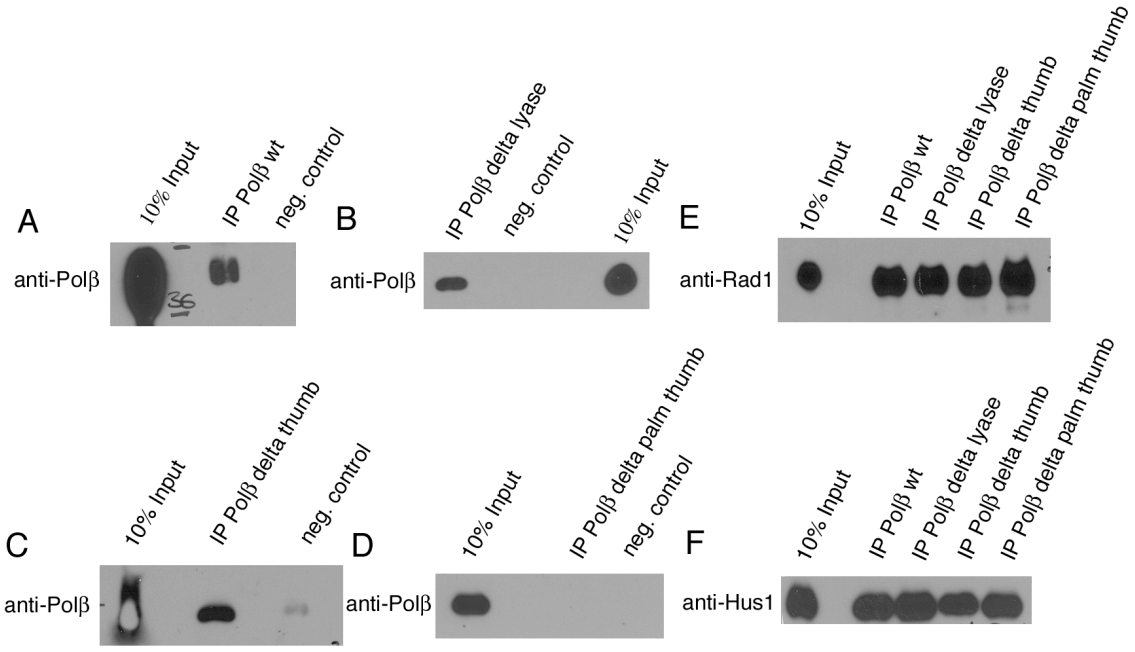
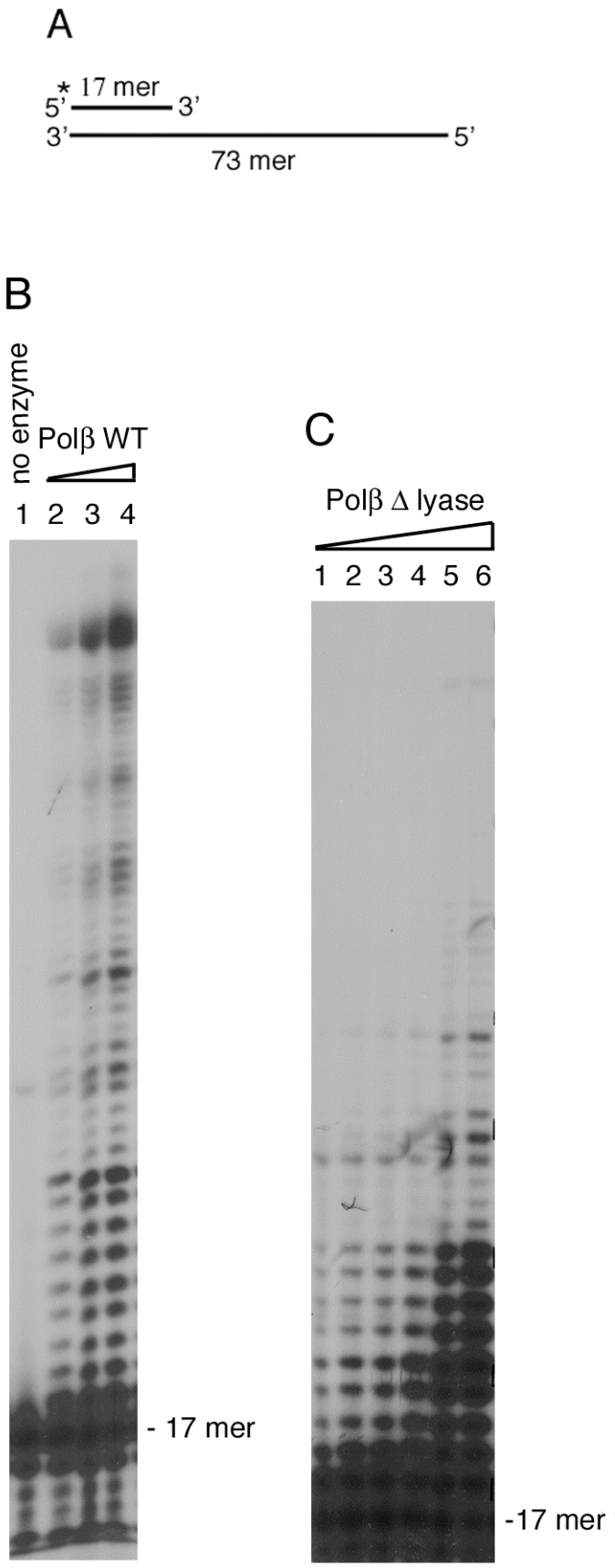


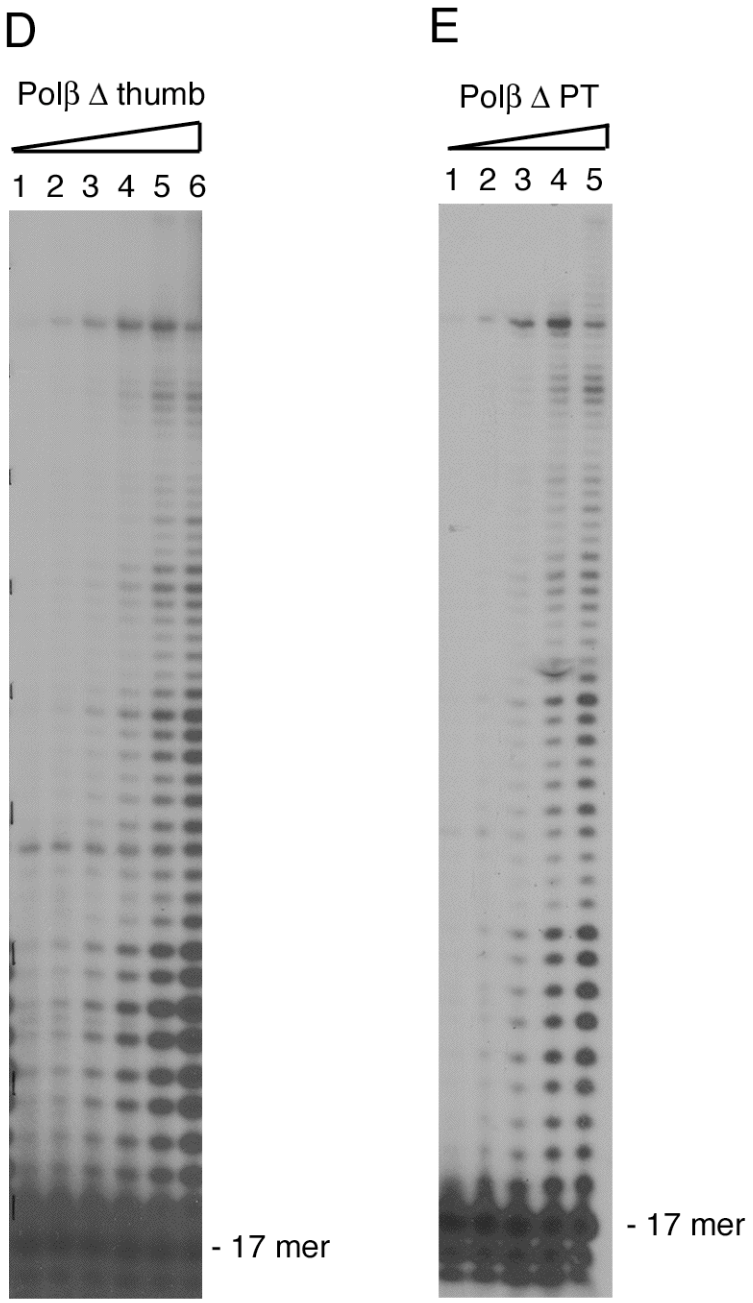
*Figure 4: DNA polymerase β likely interacts with the 9-1-1 complex via its palm-domain. Immunoprecipitation of His-DNA polymerase β wild type and the His-pol β deletion mutants by His-9-1-1 complex. (**A**) The immunoprecipitation assay was performed in the presence of 2 μl of anti-Rad9 antibody and 3.6 μg of 9-1-1 complex and 3.6 μg pol β wild type. The negative control was done by use of 2 μl of anti-Rad9 antibody with 3.6 μg DNA polymerase β only. (**B**) The immunoprecipitation assay was performed in the presence of 2 μl of anti-Rad9 antibody and 3.6 μg of 9-1-1 complex and 2.5 μg DNA polymerase β delta lyase. The negative control was done by use of 2 μl of anti-Rad9 antibody with 2..5 μg of DNA polymerase β delta lyase only. (**C**) Protein-A sepharose immunoprecipitation was performed in the presence of either 2 μl of anti-Rad9 antibody and 3.6 μg of 9-1-1 complex and 2.9 μg of DNA polymerase pol β delta thumb. The negative control was done by use of 2 μl of anti-Rad9 antibody with 2.9 μg of DNA polymerase β delta thumb only. (**D**) Immunoprecipitation was performed in the presence of 2 μl of anti-Rad9 antibody and 3.6 μg of 9-1-1 complex and 1.65 μg of DNA polymerase β delta palm thumb. The negative control was done by use of 2 μl of anti-Rad9 antibody with 1.65 μg of DNA polymerase β delta palm thumb only.*

*(**E**) The presence of the Rad1 protein in the 9-1-1 complex was tested by analyzing a part of the immunoprecipitation with anti-Rad1 antibody. (**F**) The presence of the Hus1 protein in the 9-1-1 complex was tested by analyzing a part of the immunoprecipitation with anti-Hus1 antibody. The proteins were detected by SDS-PAGE followed by Western blot (WB) analysis against the proteins indicated.*

5.5 DNA polymerase β deletion mutants harbour a decreased, but still detectable polymerase activity

To be able to later on test the influence of the 9-1-1 complex on the DNA polymerase β deletion mutants' activity, the DNA polymerase β wild type and the deletion mutants had first to be tested for their polymerase activity. This was done by a titration of the proteins in increasing amounts into the assay. As a DNA template a 5'labelled 17 mer primer annealed to a 73 mer oligonucleotide was used (schematically depicted in Figure 5, panel A). Surprisingly, all of the three mutants showed polymerase activity. However, differences in the polymerisation rate between the different mutants were observed. The DNA polymerase β delta lyase construct, having a completely conserved polymerase domain, but lacking the C-terminal 'lyase' part, was able to polymerise and accumulate mainly short products of up to 9 nucleotides in high amounts but was hardly able to elongate the strand completely (Figure 5, panel C). The total amount of products accumulated did not seem to be less than for the other mutants, but more of the short products accumulated, compensating for the lack of complete elongation activity. This suggested that this mutant has a strongly reduced processivity, in agreement with other studies on the dRP lyase domain [20].
In contrast, the DNA polymerase β delta thumb construct, lacking the thumb part of its hand-shaped polymerase domain, which enables the polymerase part to close like a fist around the DNA, elongated the primer almost completely, suggesting that it did not have lost any of its affinity to the DNA substrate (Figure 5, panel D). Already with small amounts of this mutant, a complete elongation of the primer could be observed, although the amount of synthesised products was much lower than with DNA polymerase β wild type (Figure 5, panel B). Similarly to the DNA polymerase β delta thumb mutant, also the DNA polymerase β delta palm thumb deletion mutant showed to be active without significant loss of DNA binding ability (Figure 5, panel E), but it showed an even lower level of activity compared to DNA polymerase β wild type. Since the DNA polymerase β delta palm thumb mutant lacks the active site of the polymerase catalytic site it should per definition not show any polymerase activity on its own. Therefore it is likely that this mutant was contaminated with *E. coli* DNA polymerase(s), probably due to co-purification with the mutant. For this reason the mutant was not used for further DNA polymerase activity experiments anymore.


A
* 17 mer
5'
3'
3'
5'
73 mer
B
no enzyme
Polβ WT
1 2 3 4
- 17 mer
C
Polβ Δ lyase
1 2 3 4 5 6
-17 mer



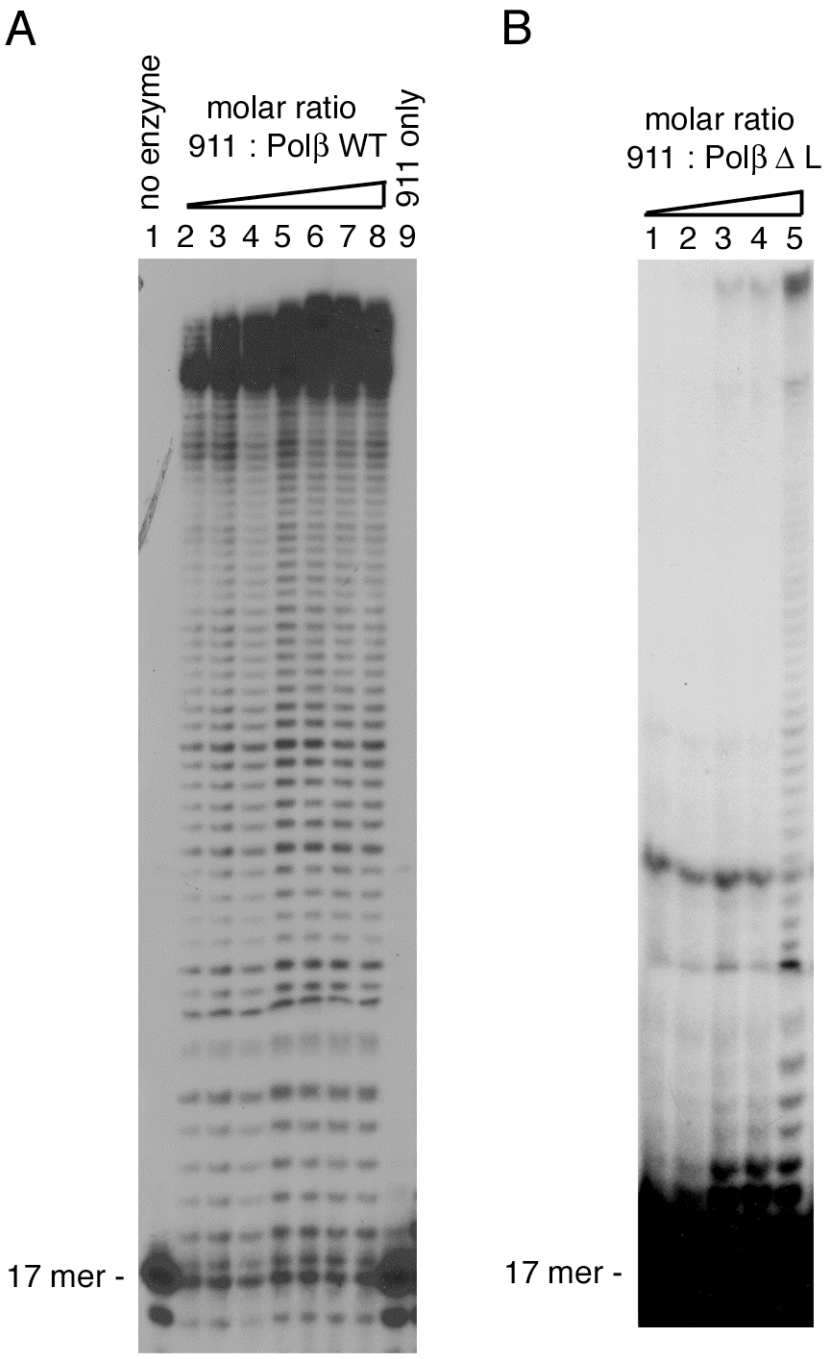
<u>Figure 5</u>: The DNA polymerase β deletion mutants display differences in polymerase activity compared to the wild type protein.
*(**A**) DNA-substrate used for the in vitro polymerase activity assays. (**B**) Titration of DNA polymerase β wild type in increasing amounts, using 50 fmol of DNA-template. No enzyme control (= lane 1) displays the signal corresponding to the template alone. Lanes 2-4 contain 0.675, 1.35 and 2.7 fmol of DNA polymerase β wild type, respectively. (**C**) Titration of DNA polymerase β delta lyase in increasing amounts, using 50 fmol of DNA template. Lanes 1-6 contain 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 nmol of DNA polymerase β delta lyase, respectively (**D**) Titration of DNA polymerase β delta thumb in indreasing amounts, using 50 fmol of DNA template. Lanes*

*1-6 contain 0.17, 0.34, 0.68, 1.3, 2.7 and 5.4 nmol of DNA polymerase β delta thumb, respectively (**E**) Titration of DNA polymerase β delta palm thumb in increasing amounts, using 50 fmol of DNA template. Lanes 1-5 contain 0.3, 0.6, 1.2, 2.4 and 4.8 nmol of DNA polymerase β delta palm thumb, respectively*

5.6 The 9-1-1 complex stimulates the activity of full length DNA polymerase β and of its mutants

To confirm the stimulating effect of the 9-1-1 complex on DNA polymerase β and to test possible effect of the 9-1-1 complex on the DNA polymerase β deletion mutants, a polymerase stimulation assay was carried out. The template used was the same as for the *in vitro* polymerase assays described in 5.4 (see Figure 5, panel A). Appropriate amounts from the linear range of the titrations (Figure 5, panels B-E) were chosen to test the effect of the 9-1-1 complex. 1.35 fmol of DNA polymerase β wild type was used and a titration of the 9-1-1 complex with increasing molar ratios was performed. As controls a constant amount of total protein in the reactions was achieved by supplementing with BSA in the according amounts (Figure 6, panel A). To rule out an eventual additional polymerase contamination by the 9-1-1 complex, the highest amount of 9-1-1 complex used for the titration was added in the absence of DNA polymerase β (Figure 6, panel A, lane 9). The molar ratios of 9-1-1 complex : DNA polymerase β were then kept the same for the two deletion mutants that were tested, but due to the weaker activity, the amounts of DNA polymerase β mutants and therefore also of the 9-1-1 complex used had to be higher. For both mutants the amount of 34 fmol was used. This represents a 25 fold higher amount than the one used for the DNA polymerase β wild type titration with the 9-1-1 complex in Figure 6, panel A. Furthermore, the ratio range of 9-1-1 complex : DNA polymerase β mutants used could not be pushed as high as for the titration with DNA polymerase β wild type, as this would have needed again too high amounts of protein. To exclude eventual contaminants due to the 9-1-1 complex used in such high amounts for the titration with the deletion mutants, a titration with the same amounts of the 9-1-1 complex in the absence of DNA polymerase β was performed. Even with such high amounts of the 9-1-1 complex used, no significant contamination by polymerases or nucleases could be detected (data not shown).

First, the stimulation of the DNA polymerase β wild type, shown already previously by work from the Institute of Veterinary Biochemistry and Molecular Biology [35], could be confirmed (Figure 5, panel A). For the DNA polymerase β delta lyase mutant (Figure 6, panel B), a clear stimulation by the 9-1-1 complex could be observed as well. The fact that it could be stimulated by the 9-1-1 complex was not really surprising when considering the data from the interaction studies, but the extent in which the loss of affinity to DNA of this mutant (shown in Figure 5, panel C) could be compensated by addition of the 9-1-1 complex was striking. By addition of the 9-1-1 complex the DNA polymerase β delta lyase mutant seems to regain at least partially the ability to perform processive polymerisation, leading to full length elongation of the DNA template. This result confirms, as shown previously in our labarotary [35], that the stimulative effect of the 9-1-1 complex triggers via an increase in the binding of the DNA polymerase β to DNA. The delta thumb mutant was stimulated by the 9-1-1 complex as well (Figure 6, panel C). The addition of the 9-1-1 complex appears to promote again an accumulation of full length products.


A
no enzyme
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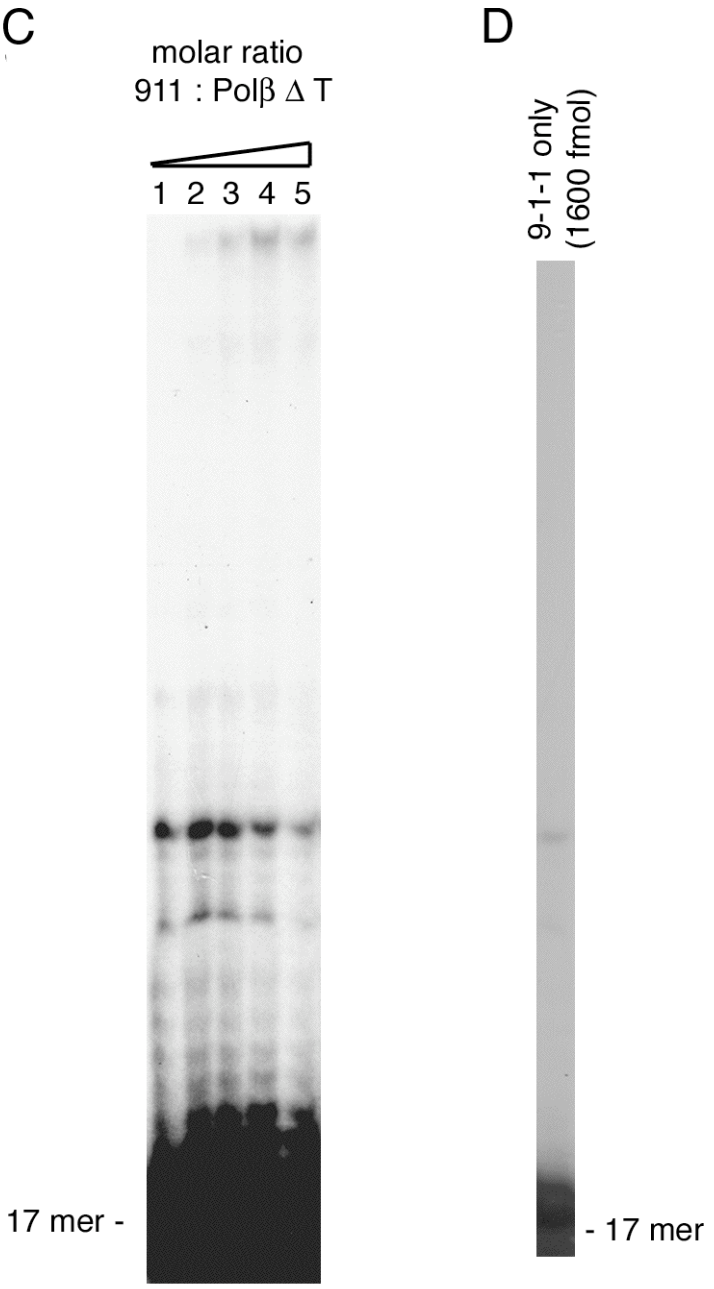


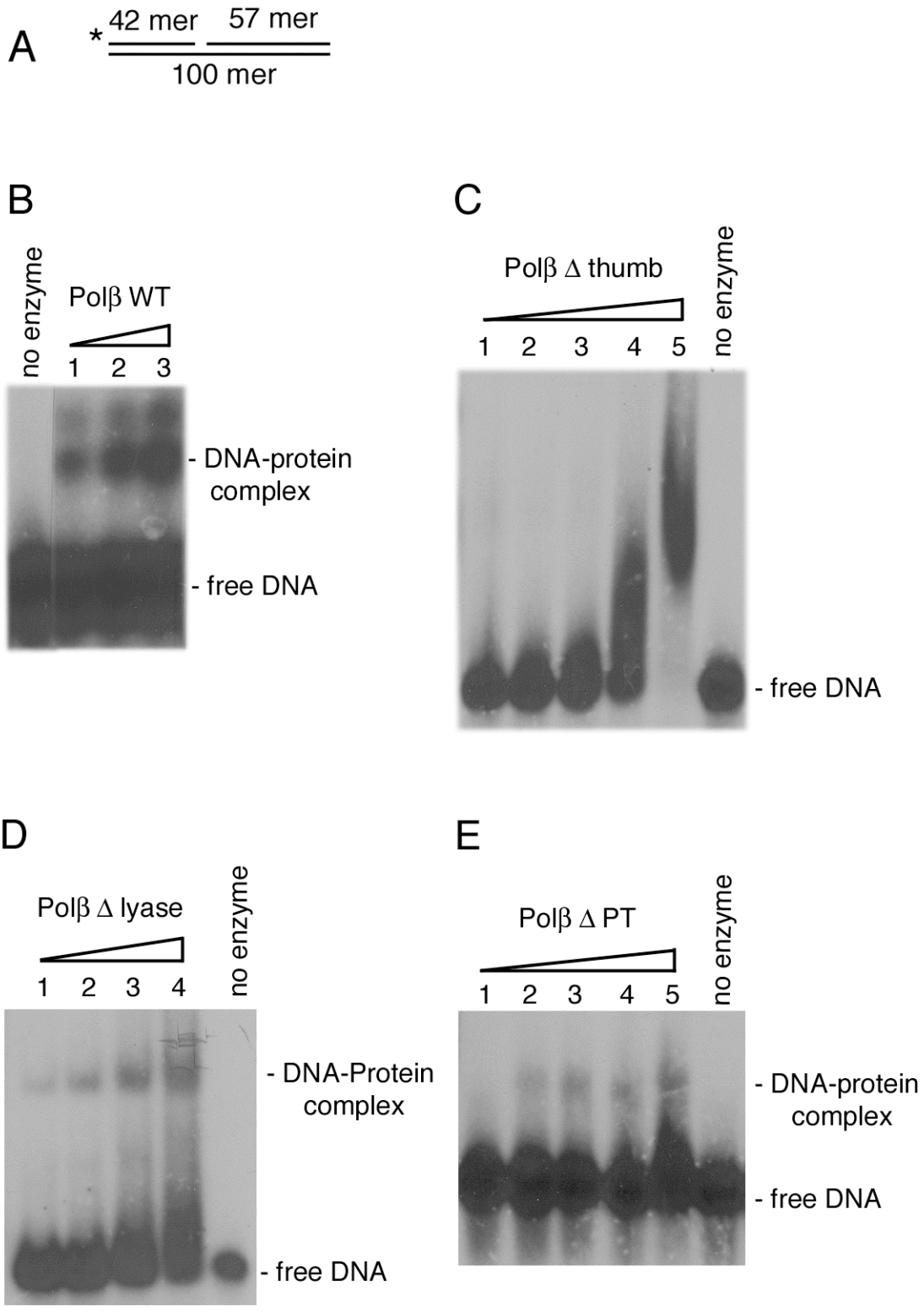
<u>Figure 6</u>: The polymerisation activity of DNA polymerase β wild type and its deletion mutants can be stimulated by the 9-1-1 complex.
*(**A**) Titration of increasing amounts of the 9-1-1 complex, always complemented with BSA to reach a constant protein amount within every tube, into a constant amount of 1.35 fmol DNA polymerase β wild type. The amounts of 9-1-1 complex that were used ranged from 0 to 880 fmol. No enzyme control (lane 1) shows the template alone, lane 2 = DNA polymerase β wild type only, lanes 3-8 = ratios of 9-1-1 complex : DNA polymerase β wild type 32, 80, 160, 320, 480 and 640, respectively. Lane 9 = 880 fmol 9-1-1 complex only. (**B**) Titration of increasing amounts of the 9-1-1 complex into a constant amount of 34 fmol of DNA polymerase β delta lyase. The reactions were always complemented with BSA to reach a constant protein amount within every tube. The amounts of 9-1-1 complex used ranged from 0 to 2720 fmol. Lane 1 = DNA polymerase β delta lyase only, lanes 2-5 = ratios of 9-1-1 complex : DNA polymerase β delta lyase 10, 20, 40 and 80, respectively. (**C**) Titration of increasing amounts of the 9-1-1 complex into a constant amount of 34 fmol of*

*DNA polymerase β delta thumb. The reactions were always complemented with BSA to reach a constant protein amount within every tube. The amounts of 9-1-1 complex used ranged from 0 to 2720 fmol. Lane 1 = DNA polymerase β delta thumb only, lanes 2-5 = ratios of 9-1-1 complex : DNA polymerase β delta thumb 10, 20, 40 and 80, respectively. (**D**) Negative control for the 9-1-1 complex in high amounts used as in the titrations for the mutants. The amount of the 9-1-1 complex that was used for this negative control corresponds to a ratio of 9-1-1 complex : DNA polymerase β mutant of 50. Exposition time like for the films for the mutant titrations. This negative control shows, that there is not significant polymerase or nuclease contamination in the 9-1-1 complex batch.*

5.7 DNA polymerase β's binding to DNA is greatly decreased in the absence of the lyase domain

To address the question, whether the reduced processivity of the delta lyase mutant might be due to a reduction of its binding ability to DNA, an EMSA (Electrophoresis mobility shift assay) using a 1 nucleotide gap oligonucleotide (depicted in Figure 7, panel A) was performed with the wild type protein and each of the mutants. The DNA polymerase β wild type and its mutants were titrated in increasing amounts into the reaction.

The results revealed, that DNA polymerase β delta thumb (Pol β ΔT, Figure 7, panel C) and DNA polymerase β delta palm thumb (Polβ ΔPT, Figure 7, panel E) were able to bind to DNA almost equally to DNA polymerase β wild type (Polβ WT, Figure 7, panel B) although the observed band shift was slightly weaker when comparable amounts of the mutants were used. However, the DNA polymerase β delta lyase (Polβ ΔL, Figure 7, panel D) showed a strongly reduced ability to bind to DNA. Molar amounts that had to be used for the DNA polymerase β delta lyase mutant to see a comparable DNA-bandshift are 150 times higher than the amounts used for the DNA polymerase β delta thumb and DNA polymerase β delta palm thumb mutants. This result confirms the data obtained in the polymerase assay for the DNA polymerase β delta lyase mutant, which was not able to synthesize full length products and this is likely due to its reduced DNA binding ability.



*<u>Figure 7</u>: DNA polymerase β's binding to DNA is decreased in the absence of the lyase domain. An electrophoresis mobility shift assay with 50 fmol of 1 nt gap DNA substrate and increasing amounts of DNA polymerase β wild type and its mutants was performed. (**A**) 1 nt gap 5'labelled DNA substrate used for the EMSA. (**B**) Titration of DNA polymerase β wild type in increasing amounts into the reaction with 50 fmol DNA substrate. The no enzyme control is the reaction mix without adding DNA polymerase β.*

*Lanes 1-3: 35, 70 and 140 fmol of DNA polymerase β wild type, respectively. The result shows increased affinity of DNA polymerase β wild type to the DNA substrate with increasing DNA polymerase β wild type amounts used. (**C**) Titration of DNA polymerase β delta thumb in increasing amounts into the reaction with 50 fmol DNA substrate. The no enzyme control is the reaction mix without adding DNA polymerase β delta thumb. Lanes 1-5: 34, 68, 170, 340 and 680 fmol of DNA polymerase β delta thumb, respectively. The result shows increased affinity of DNA polymerase β delta thumb to the DNA substrate with increasing DNA polymerase β delta thumb amounts used. (**D**) Titration of DNA polymerase β delta lyase in increasing amounts into the reaction with 50 fmol DNA substrate. The no enzyme control is the reaction mix without adding DNA polymerase β delta lyase. Lanes 1-4: 10, 20, 40 and 80 nmol of DNA polymerase β delta lyase, respectively. The result shows increased affinity of DNA polymerase β delta lyase to the DNA substrate with increasing DNA polymerase β delta lyase amounts used, but also clearly an greatly decreased affinity for the DNA template compared to the other DNA polymerase β EMSA titrations. (**E**) Titration of DNA polymerase β delta palm thumb in increasing amounts into the reaction with 50 fmol DNA substrate. The no enzyme control is the reaction mix without adding DNA polymerase β delta palm thumb. Lanes 1-5: 34, 68, 170, 340 and 680 fmol of DNA polymerase β delta palm thumb, respectively. The result shows increased affinity of DNA polymerase β delta palm thumb to the DNA substrate with increasing DNA polymerase β delta palm thumb amounts used.*

5.8 DNA polymerase β's binding to DNA is enhanced in presence of the 9-1-1 complex

In order to confirm the stimulative effect of the 9-1-1 complex on DNA polymerase β's ability to bind to its DNA substrate, EMSA's with a titration of the 9-1-1 complex in increasing amounts into a constant amount of DNA polymerase β wild type and its mutants (chosen according to the titrations in Figure 7) were performed. First, a titration of the 9-1-1 complex only in the amounts used later on in the assays was done, to confirm that the 9-1-1 complex alone does not induce any DNA bandshifting (Figure 8, panel A). By using 35 fmol of the DNA polymerase β wild type and titrating in the 9-1-1 complex, the stimulative effect of the 9-1-1 complex on DNA polymerase β wild type's binding to DNA could clearly be confirmed by seeing an enhanced intensity of the DNA bandshift with increasing ratios of the 9-1-1 complex (Figure 8, panel B), resulting from enhanced binding of DNA polymerase β wild type to DNA. A band that is even higher than the primary bandshift was appearing, indicating that an additional complex might have been formed.

By using the same molar 9-1-1 complex : DNA polymerase β ratios for the deletion mutants, only the stimulative effect of the 9-1-1 complex on the two mutants DNA polymerase β delta thumb and DNA polymerase β delta palm thumb could be tested, due to the very weak binding ability to DNA of the DNA polymerase β delta lyase mutant (see Figure 7, panel D). The use of such high amounts of protein (namely the 9-1-1 complex and the DNA polymerase β delta lyase) to do the titration in a similar ratio as for the other 2 mutants would have given not more than an unclear

smear of proteins on the gel without any possibility to draw conclusions from such results. Therefore the stimulation of this activity could not be tested for the DNA polymerase β delta lyase mutant.

For the DNA polymerase β delta palm thumb mutant, a weak, but still visible increasing DNA bandshift was observed, correlating to the increasing amount of the 9-1-1 complex used in the assay (Figure 8, panel C). This was a surprising result, as the immunoprecipitation of DNA polymerase β delta palm thumb by the 9-1-1 complex never showed any interaction. Still, this mutants' binding to DNA could be weakly stimulated, suggesting, that a possible weaker interaction site of the 9-1-1 complex with the DNA polymerase β might exist on the palm subunit as well.

For the DNA polymerase β delta thumb mutant, however, a good quality picture to show the increase of DNA bandshift could never be produced. This was likely due to the fact, that the shifted band migrated too close to the template to be distinguished properly when reasonable amounts of this mutant were used.

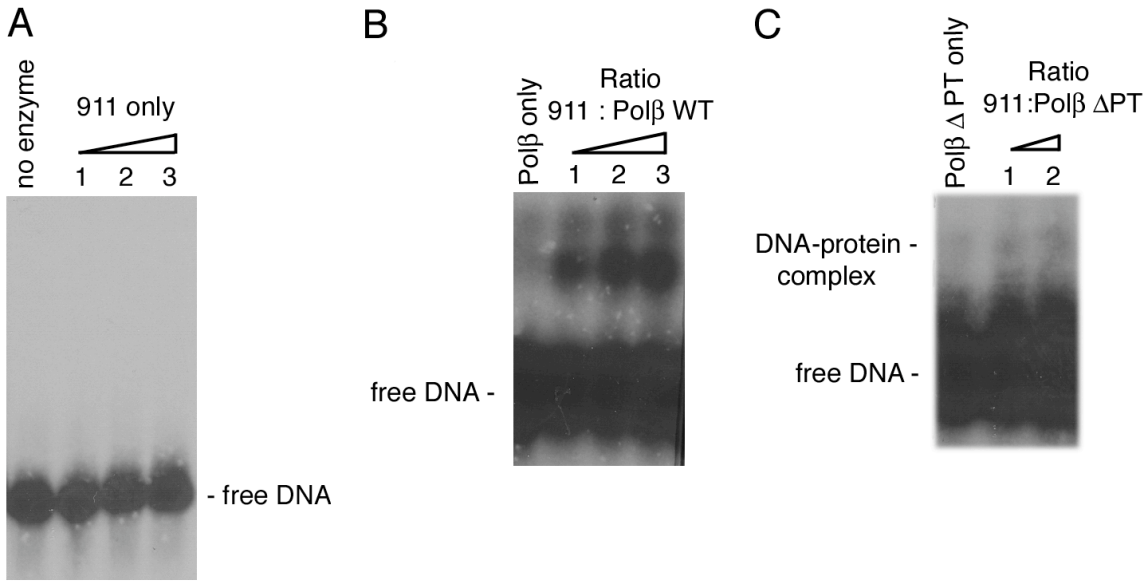


Figure 8: DNA polymerase β and its delta palm thumb mutants binding ability to DNA is enhanced in the presence of the 9-1-1 complex
*An Electrophoresis mobility shift assay was performed with DNA polymerase β wild type and delta palm thumb mutant to detect increased binding to DNA in presence of the 9-1-1 complex. (**A**) A titration of increasing amounts of the 9-1-1 complex to 50 fmol of DNA template was performed to exclude any bandshift due to the 9-1-1 complex alone. The amounts that were tested here were used later on in the titrations for DNA polymerase β wild type and, with exception of the highest amount, also for the delta palm thumb mutant. The no enzyme control shows the reaction mix only without addition of the 9-1-1 complex. Lanes 1-3: 0.5, 1 and 2 nmol of 9-1-1 complex, respectively. (**B**) Titration of the 9-1-1 complex in increasing amounts into 35 fmol of DNA polymerase β wild type and 50 fmol of DNA template. Lanes 1-3 show the following ratios of 9-1-1 complex : DNA polymerase β wild type: 15, 30, 60. A clear enhancement of the DNA bandshift can be observed on addition of increasing amounts of the 9-1-1 complex. DNA polymerase β only shows the bandshift induced without addition of 9-1-1 complex. (**C**) A titration of the 9-1-1 complex in increasing amounts into 100 fmol of DNA polymerase β delta palm thumb*

and 50 fmol of DNA substrate. Polβ Δ PT only shows the bandshift induced without addition of 9-1-1 complex. Lanes 1and 2 show the following ratios of 9-1-1 complex : DNA polymerase β wild type: 15 and 30. A slight DNA bandshift can be observed with increasing amounts of the 9-1-1 complex.

5.9 Putative binding motif(s) of for the 9-1-1 complex on DNA polymerase β

Taken together the facts that a binding site for the 9-1-1 complex is existing on the C-terminal part (amino acids 360-380) of Fen1 [36], on human DNA ligase I [45] and on Rad-17 [32] as well as on DNA polymerase β [35], the next aim was to look for a common possible specific binding motif present on these four proteins. An alignment of sequences of human DNA ligase I, DNA polymerase β (finger and palm domain), Rad17 and Fen1 (N-terminal part, amino acids 360-380), was performed, by using the ClustalW program for protein sequence alignment from the European Bioinformatics Institute. After the detection of putative conserved sequences, APE1 as a further putative 9-1-1 complex binding partner was also tested for the presence of such a site. The results were the following:

- DNA polymerase β, Fen1, Rad17 and APE1 share a sequence of mainly 6 amino acids, that show a conserved glycine and other highly conserved or semi-conserved amino acids (Figure 9, sequence coloured **cyan**).
- DNA ligase I, DNA polymerase β, Rad17, APE1 and Fen1 share a sequence of again 6 amino acids, displaying high conservation of the amino acids (Figure 9, sequence coloured **blue**).
- DNA polymerase β, Fen1, Rad17, DNA ligase I and APE1 display a sequence of 8 amino acids, that are conserved to a wide extent. (Figure 9, sequence coloured **pink**)

It is interesting, that for Fen1 the first (cyan) and the second (blue) motif are partly overlapping, possibly forming a single binding site. This is also the case for APE1, in which the first (cyan) sequence incorporates completely the second (blue) sequence. Furthermore one could argue that the probability of finding a novel 9-1-1 interaction partner in APE1 is quite high.

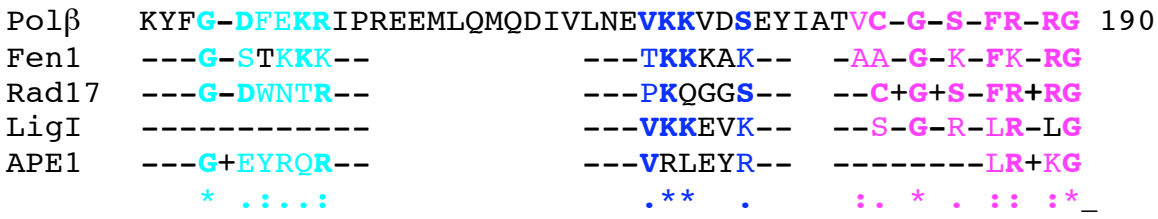
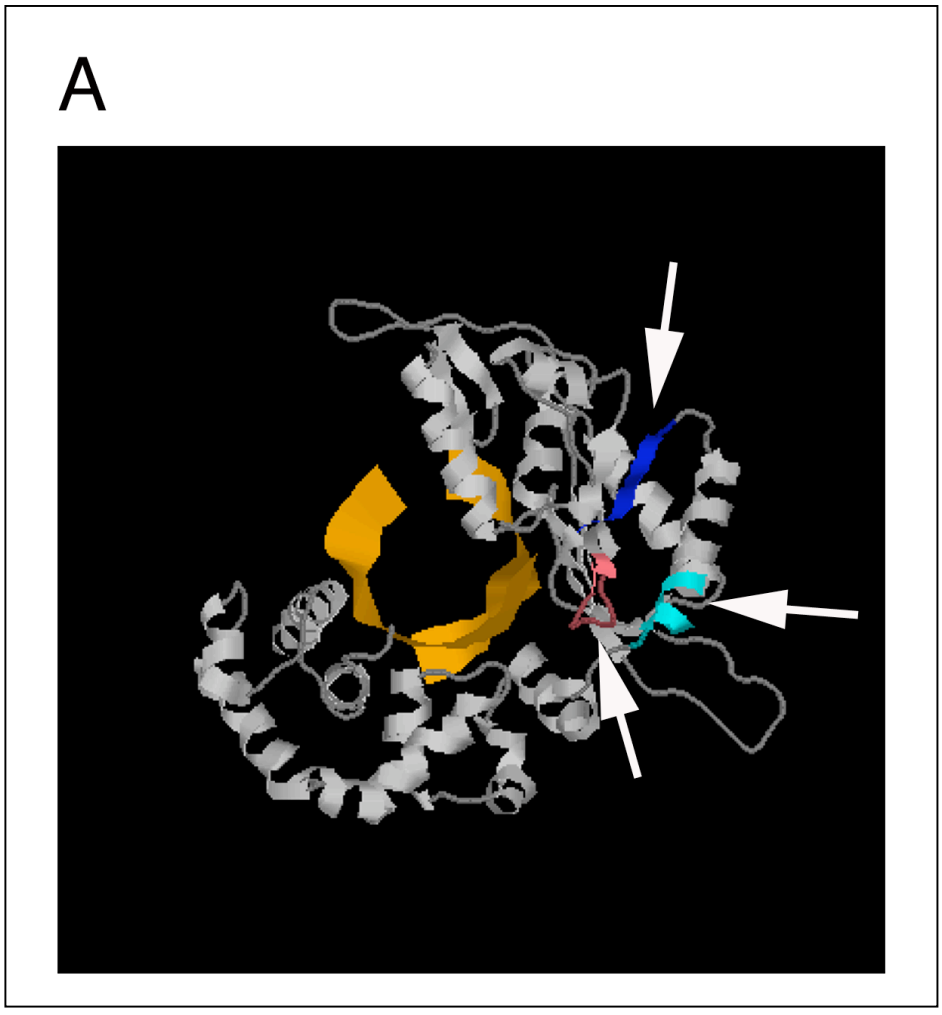


Figure 9: Sequence alignment showing amino acids 141 – 190 of DNA polymerase β, aligned to the putative conserved sequences of Fen1, Rad17, DNA ligase I and APE1 showing possible 9-1-1 complex binding motifs. A "+" indicates that there are several amino acids present in the sequence, which are not displayed, presumably forming loop-like structures outside of the binding motiv. A "" indicates indentical amino acids, a ":" shows highly conserved amino acids and a "." points out semiconserved substitutions (more information to this classification available on the website of the European Bioinformatics Institute) The amino acids marked in **bold** show the amino acids in the different sequences that are identical to the DNA polymerase β sequence. (**CYAN**) This first putative binding motif is conserved on DNA polymerase β (AA 144 – 149), Fen1 (AA 362 – 367), Rad17 (AA 471 – 476) and APE1 (AA 178 – 187). (**BLUE**) The second putative binding motif is located on DNA polymerase β (AA 166 – 171), Fen1 (AA 364 – 369), Rad17 (AA 115-120), DNA ligase I (AA 239 – 244) and APE1 (AA 180 - 185) (**PINK**). This third conserved sequence can be found on DNA polymerase β (AA 177 – 184), Fen1 (AA 372 – 379), Rad17 (AA 503 – 515), DNA ligase I (AA 447 – 457) and on APE1 (220 – 225).*

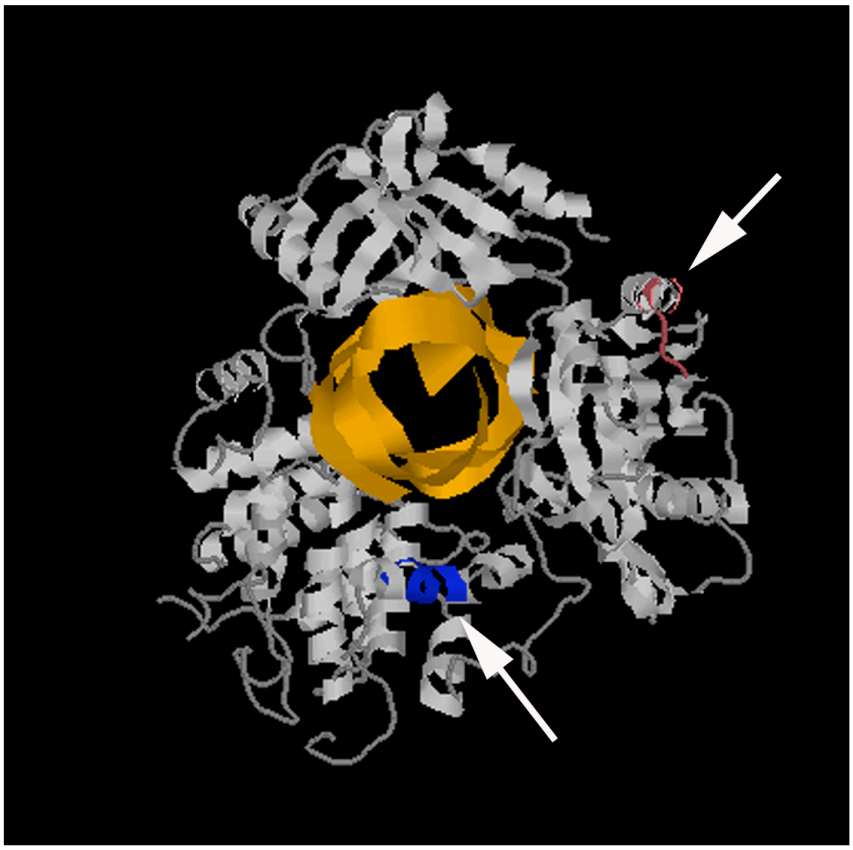
The existence of 2 or 3 putative binding motifs for the 9-1-1 complex on all the proteins known or believed to interact with the 9-1-1 complex is exciting, as for example Fen1 and DNA polymerase β, which obviously display all the three binding motifs, have been shown to interact with all the 3 subunits of the 9-1-1 complex. This existence of 3 binding motifs would be an explanation that all the 3 subunits can bind, as they do not dispose of consensus sequences, which rules out most probably a single binding site for all the 3 subunits together.

5.10 The three putative 9-1-1 complex binding motifs are located on the outer surface of DNA polymerase β and DNA ligase I

The three putative binding motifs were tested for their location on the structural model of DNA polymerase β, on DNA ligase 1 and on APE1, respectively. Unfortunately, for Rad17 there is no structure model existing yet, and for Fen1, none including the last 20 amino acids, which are the relevant part for the interaction with the 9-1-1 complex. Therefore those two highly interesting proteins could not be investigated for structural location of those sites. However, the structural investigation of DNA polymerase β wild type revealed, that the three motifs are located closely to each other on the outer surface of the protein (Figure 10, panel A; the coloured motifs are also pointed at with an arrow). Moreover, they seem to be orientated towards the side of the protein, where the DNA, which is encircled by DNA polymerase β, leaves the protein. This seems to be a logical location for a binding site, as the 9-1-1 complex closes around the DNA as a ring, probably giving the recruited proteins the opportunity to attach to it near the DNA passing through the ring, as depicted schematically on Figure 12. For DNA ligase I, a similar observation could be made (Figure 10, panel B). The putative two binding motifs are located on the same face of the DNA ligase I as on the DNA polymerase β, always close to the site, where the DNA strand leaves the protein. Finally, APE1 seems to display a location of the motifs very much like DNA polymerase β and DNA ligase I (Figure 10, panel C). Here the blue, second motif is not coloured specifically, as it is located in the middle of the cyan, first motif, and therefore is already covered by the cyan colouring.



B



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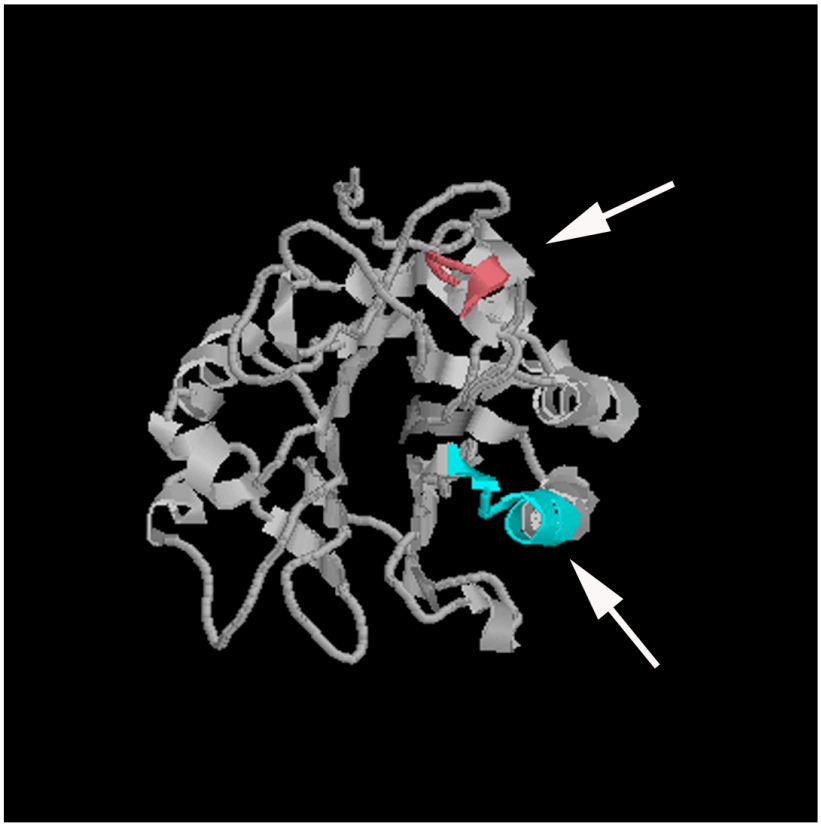

*Figure 10: Putative 9-1-1 complex binding motifs on DNA polymerase β (A), DNA ligase I (B) and APE1 (C). (**A**) DNA polymerase β wild type displays the putative binding motifs on its outer surface and in close proximity to each other. The motifs are located very closely to the surface on which the DNA strand that is bound leaves the protein. The sequences are coloured according to the alignment in Figure 9. (**B**) DNA ligase I crystal structure with the putative binding motifs coloured according to the alignment in Figure 9. The location of the motifs is close to the side of the protein, where the DNA strand, that is bound, leaves the protein again. (**C**) APE1 structural model with coloured putative binding motifs, according to Figure 9.*

To conclude the investigations so far, it was finally interesting to see, how the distribution of those putative 9-1-1 complex binding motifs compares to the known PCNA binding motifs (reviewed in [50]). Figure 11 shows a schematic representation of the five proteins. In all the cases where a PCNA binding motif was identified (DNA polymerase β, DNA ligase I and Fen1) the PCNA binding motif is located on at different site suggesting that the two clamps (PCNA and the 9-1-1 complex bind to different site on their partners. These motifs are now the bases to carry out site directed deletion mutagenesis to find out whether proteins binding to 9-1-1 complex also have a common motif to bind to their partners.

Distribution of binding motifs

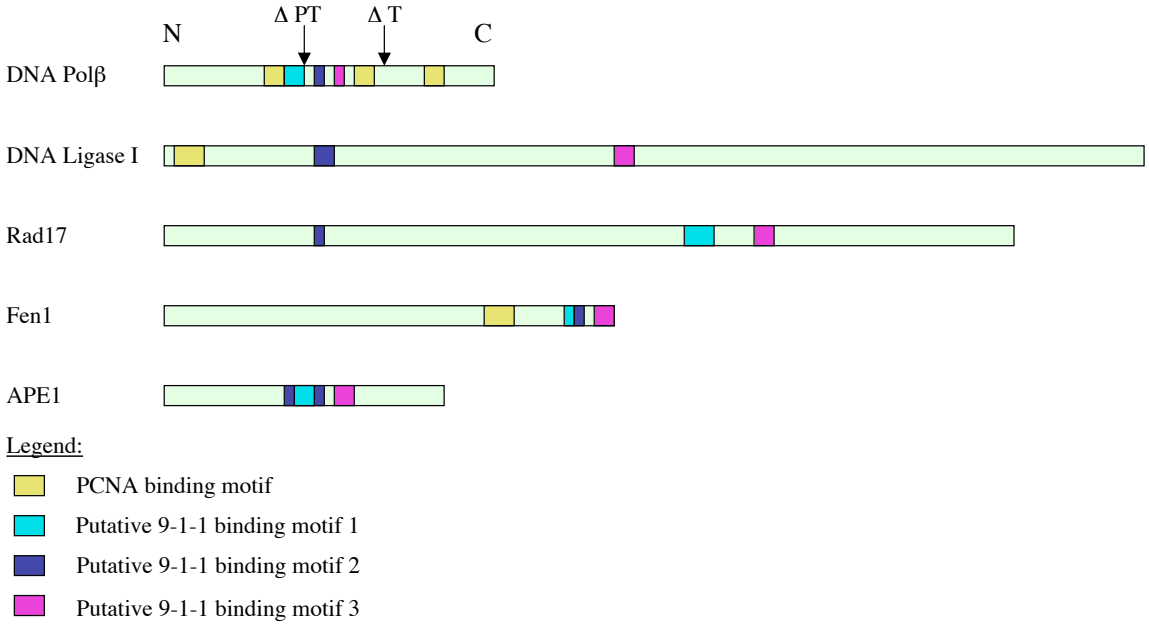

Figure 11: Schematic presentation of the location of the known PCNA binding motif and the putative 9-1-1 complex binding motifs on DNA polymerase β, DNA ligase I, Rad17, Fen1 and APE1. The sequences refer all to human proteins. The colours for the 9-1-1 complex binding motifs are

correlating to the coloured sequences in Figure 9 and all the colour codes used are indicated in the figure legend. The sequences are depicted in approximate length correlation to each other and the motifs are placed on the sequences in approximate spatial distribution.

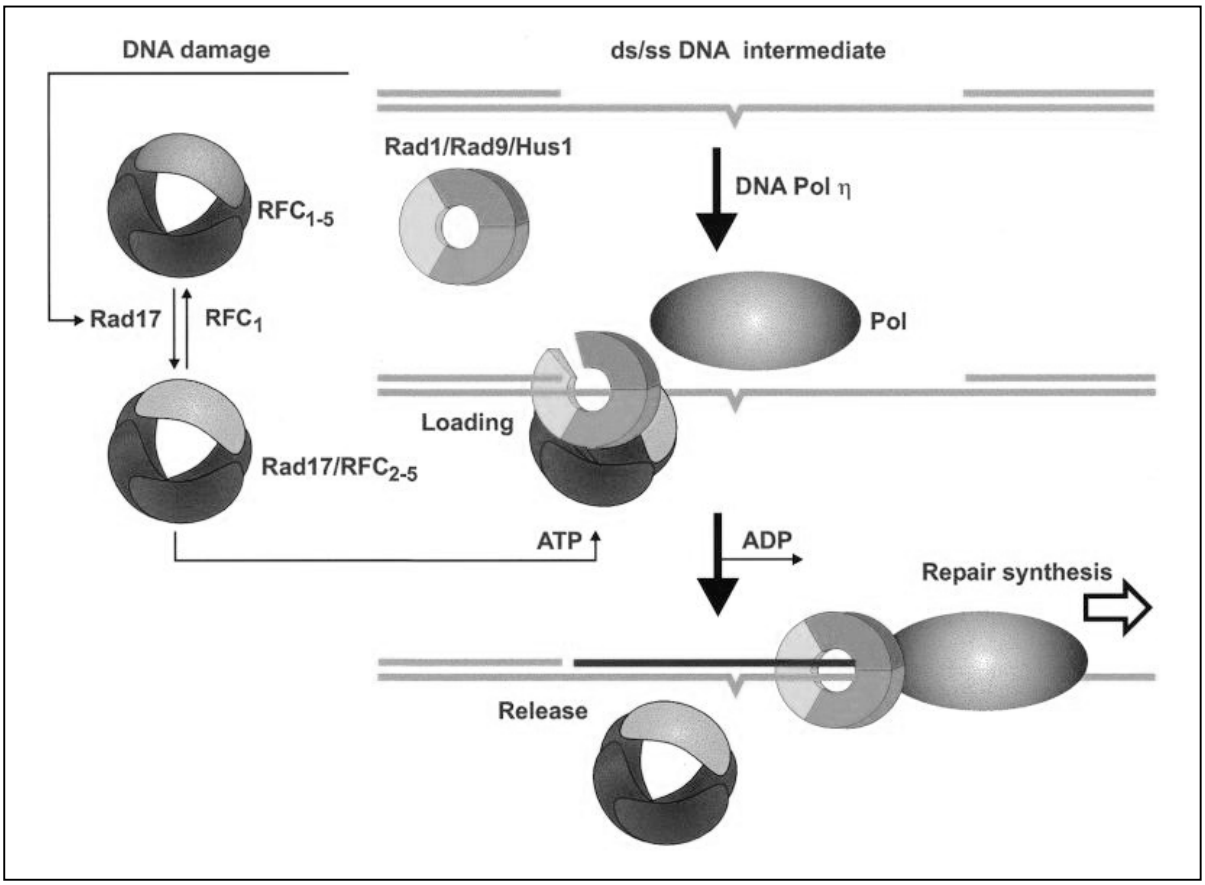

*Figure 12: Proposed mechanism of Rad1, Rad9, Hus1 and Rad17 action in response to DNA damage. The DNA intermediate contains an unrepaired damaged site, (denoted by a bulge in the lower strand), that initiates translocation of Rad17 throughout the nucleus. Conversion of RFC1–5 to Rad17/RFC2–5 is required for ATP-dependent loading of the Rad1/Rad9/Hus1 heterotrimeric ring onto DNA. Repair synthesis can then proceed by (for example) a lesion bypass polymerase that utilizes the heterotrimeric ring for more efficient and/or accurate synthesis. (Reproduction from: "**Structure-based predictions of Rad1, Rad9, Hus1 and Rad17 participation in sliding clamp and clamp-loading complexes",** C. Venclovas and M. P. Thelen, [27])*

6 Discussion

The 9-1-1 complex has been shown to be recruited onto DNA following DNA damage as an early step of the checkpoint cascades [52]. Its structural similarity to PCNA suggested that it might act as an alternative sliding clamp for certain DNA polymerases, such as repair or translesion DNA polymerases (Figure 12), [25-28]. Recent data suggested that the 9-1-1 complex may play a role in recruitment of proteins involved in BER [33,34]. The aim of this thesis was to analyse in details the interaction of the 9-1-1 complex with the two BER proteins DNA polymerase β and DNA ligase I.

A first step in this work was to characterise the DNA polymerase β mutants used for the interaction studies. This characterisation led to several interesting findings. For the delta lyase mutant it was astonishing to see that, even though it contained the full part of the polymerase domain which is shown to be responsible for the entire polymerisation process on DNA templates, it seems to be hardly able to polymerise longer products than approximately 9 nucleotides. This observation is in strong contrast to the fact, that the wild type protein in a 200 fold smaller amount elongates easily the entire template strand. It seems, that the lacking lyase part of the protein is very important for mediation of the binding to DNA, which, when not present, causes a decrease in DNA polymerase β's affinity to the DNA template. Since the intensity of the signal of the accumulated small products seems to be much higher than the one compared to the other mutants that have conserved their processivity, it can be concluded that the polymerase-activity per se is not hindered or diminished. Obviously this mutant has lost its ability to bind properly to the DNA and may stay attached to it, which is a major issue when regarding the processivity of DNA polymerase β. Further evidence for this is also provided by the fact, that in DNA-binding experiments with EMSA, a minimal 150 fold higher amount of DNA polymerase β delta lyase compared to the other mutants had to be used to be able to observe any significant bandshift.

Other observations concern the two polymerase-domain mutants, which were initially not expected to be as active as observed in the activity assays. Specially the activity of the DNA polymerase β delta thumb mutant was quite astonishing, while the fact, that the DNA polymerase β delta palm thumb mutant, lacking the catalytic site for polymerase activity, showed polymerase activity as well can most probably be explained by a contamination of this batch of protein by *E.coli* DNA polymerases. It would be of great interest to test the DNA polymerase β delta thumb mutant for its mismatch incorporation activity, as it has been shown, that the thumb domain is responsible for correct nucleotide pairing by shifting the protein from an 'open' conformation to a 'closed' one, when the correct nucleotide to be incorporated is present [51]. It could be, that the delta thumb mutant incorporates just randomly nucleotides on the growing DNA strand, no matter what nucleotide is located opposite the incorporation site.

The comparison between the physical and functional interaction of the 9-1-1 complex with DNA polymerase β and its deletion mutants yielded to some rather puzzling results. After obtaining the results from the immunoprecipitation assays, that the 9-1-1 complex interacted with the palm subdomain, the question about the binding site of the 9-1-1 complex to DNA polymerase β seemed to be quite clearly answered. In all the repetitions of this assay, an interaction between the delta palm thumb mutant and the 9-1-1 complex could never be observed, while the 9-1-1 complex could be

immunoprecipitated by the other two mutants and the wild type protein. Nevertheless, the data received from the DNA-binding stimulation assays with EMSA's seemed to contradict the immunoprecipitation data. Here it became obvious, that all of the tested mutants, including the delta palm thumb mutant, could be stimulated by the 9-1-1 complex specifically, even though in lower range. To explain this, the idea of multiple different binding sites of the 9-1-1 complex on the DNA polymerase β, of which a stronger one is located on the palm domain and another weaker one is located on the finger domain, was proposed. Our hypothesis was that, if the stronger interaction site is not present anymore in the protein (which would be then the case when using the palm thumb mutant), the interaction is probably more transient and cannot necessarily be detected in the immunoprecipitation experiments. However, the binding of the 9-1-1 complex to the weaker interaction motif could still be strong enough to stimulate DNA polymerase β's DNA binding activity. Another hint towards this hypothesis was, that the bandshift caused by the 9-1-1 complex to the delta palm thumb mutant seems to be much weaker than the one observed in the wild type, not perfectly explainable only by the lower activity of DNA binding of this mutant. The existence of such multiple binding sites would explain both, a loss of interaction when immunoprecipitating DNA polymerase β delta palm thumb by the 9-1-1 complex through the loss of the strongly interacting binding motif, but a conserved stimulating influence on the DNA binding activity via the weaker and less conserved interaction motif. To investigate this possibility, a sequence alignment was performed, comparing the sequences of the five 9-1-1 complex binding proteins DNA ligase 1, DNA polymerase β (fingers and palm domain), Fen1 (N-terminal part consisting of the last 20 amino acids, shown to be the part interacting with the 9-1-1 complex [36]), APE 1 (as putative partner for the 9-1-1 complex) and Rad17. The data obtained from the sequence alignment showed three conserved putative binding motifs, with two of them located on the palm domain and a third one located on the finger domain.

These findings were then combined with structural analysis of the crystal structure of DNA polymerase β, DNA ligase I and APE1, in order to see where those putative interaction sites were located on the proteins. It was evident, that the predicted putative 9-1-1 complex binding sites found by sequence alignment are located on exposed areas of the outer surface of each of the proteins, and therefore also easily accessible to other proteins. The sequences were then compared further to the location the PCNA binding motif, and in none observed cases they overlapped.

In conclusion, in order to further investigate those possible interaction sites, it would be necessary to perform site-directed mutagenesis. By mutating important amino acids placed within a relevant binding motif, a loss of interaction could be observed, which would confirm the physiological relevance of those so far putative interaction motifs.

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8 Original publication

Smirnova, E. Toueille, M., **Markkanen, E**. and Hübscher, U.:
The human checkpoint sensor and alternative DNA clamp Rad9–Rad1–Hus1 modulates the activity of DNA ligase I, a component of the long-patch base excision repair machinery***,***
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My contribution to this work was the physical interaction study between DNA ligase I and the 9-1-1 complex and is represented in Figure 1 of this paper.

ACCELERATED PUBLICATION

The human checkpoint sensor and alternative DNA clamp Rad9–Rad1–Hus1 modulates the activity of DNA ligase I, a component of the long-patch base excision repair machinery

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The human checkpoint sensor and alternative clamp Rad9–Rad1–Hus1 can interact with and specifically stimulate DNA ligase I. The very recently described interactions of Rad9–Rad1–Hus1 with MutY DNA glycosylase, DNA polymerase β and Flap endonuclease 1 now complete our view that the long-patch base excision machinery is an important target of the Rad9–Rad1–Hus1 complex, thus enhancing the quality control of DNA.

Key words: DNA glycosylase, DNA ligase I, DNA polymerase β, Flap endonuclease 1 (Fen1), proliferating-cell nuclear antigen (PCNA), Rad9–Rad1–Hus1 complex (9-1-1 complex).

INTRODUCTION

The mammalian genome undergoes approx. 100000 modifications per day. DNA base damage generated by ionizing radiation and simple alkylating agents, as well as by endogenous hydrolytic and oxidative processes, is corrected by the BER (base excision repair) pathway [1]. Two BER sub-pathways have been characterized using *in vitro* and *in vivo* methods, and have been classified according to the length of the repair patch as either 'short-patch' BER (SP-BER, one nucleotide) or 'long-patch' BER (LP-BER; more than one nucleotide) [2]. The first step in both BER sub-pathways is carried out by DNA glycosylases [examples being OGG1 (8-oxoguanine DNA glycosylase 1), NTH1 (Nth endonuclease III homologue 1) and MYH (MutY human homologue)], which are specific for a particular type of base damage or, more commonly, a group of related types. These enzymes remove the damaged bases, leaving potentially mutagenic AP (apurinic/apyrimidinic) sites. A resulting abasic site is then recognized by APE1 [AP endonuclease 1, also known as HAP1 (human apurinic endonuclease 1)], which incises the damaged strand, leaving a single-nucleotide gap with 3′-OH and 5′-dRP (5′-deoxyribose phosphate) groups flanking the nucleotide gap. Further repair can be accomplished via two pathways that involve different subsets of enzymes and result in replacement of one (SP-BER pathway) or two to ten (LP-BER pathway) nucleotides. In mammalian cells, SP-BER is considered to be the major BER pathway, whereas LP-BER is an important back-up pathway [3].

In the LP-BER pathway, the collaboration of DNA pol (polymerase) δ/ε, PCNA (proliferating-cell nuclear antigen), RF-C (replication factor C) and Fen1 (Flap endonuclease 1) can displace the 5′ nick and synthesize up to ten nucleotides. The flap is cut by Fen1, and the final nick is sealed by DNA ligase I [4,5]. Both pols δ and ε can participate in this reaction [6], and DNA ligase I is likely to be the patch size mediator in LP-BER [7]. The same protein components are also required for *in vitro* reconstitution of the LP-BER pathway for 7′8′-dihydro-8-oxoguanine [8]. On the other hand, the LP-BER pathway can also be carried out by pol β, Fen1 and DNA ligase I. Here, pol β, with its strand displacement synthesis, and Fen1 co-operate with the so-called 'hit-and-run' mechanism [9]. Finally, it was found that the tumour-suppressor protein APC (adenomatous polyposis coli) can block the strand-displacement synthesis by pol β during LP-BER, thus increasing the sensitivity to methylmethane sulphonate [10]. The LP-BER proteins DNA glycosylase, HAP1, pol β, Fen1 and DNA ligase I can all interact physically with the PCNA clamp [11].

On the other hand, checkpoints are activated upon DNA damage in eukaryotic cells in order to stop cell-cycle progression. This activation requires the action of DNA-damage sensors and transducers [12]. Among these, the three human proteins Rad9, Hus1 and Rad1 form a heterotrimeric complex (called the 9-1-1 complex) exhibiting structural similarity with the homotrimeric clamp PCNA [13,14] that can be loaded on to DNA by the Rad17–RF-C_{2-5} clamp loader [15]. Moreover, the 9-1-1 complex, Rad17–RF-C_{2-5} and PCNA co-localize in foci formed upon DNA damage [16,17]. These data suggested a mechanism in which Rad17–RF-C_{2-5} would localize on DNA lesions, allowing the recruitment of the 9-1-1 complex to these sites. Subsequently, the 9-1-1 complex would serve as a recruiting platform for the checkpoint effector kinases such as Chk1 or Chk2, which are subsequently phosphorylated by the ATR [ATM (ataxia telangiectasia mutated) and Rad3-related]/ATM kinases [18]. Additionally, a model has recently been proposed by two different groups, where the 9-1-1 complex and the Rad17–RF-C_{2-5} clamp loader could stabilize stalled replication forks [19,20].

The mechanisms by which DNA repair occurs are now quite well unravelled [1]. Likewise, the way that cells trigger the DNA-damage checkpoints is also starting to be deciphered [18]. However, the link between checkpoint engagement and the recruitment to DNA lesions of repair machineries is far from being understood. Recent studies performed in yeast have shown interaction or co-localization of the alternative checkpoint clamp, the 9-1-1 complex, with proteins involved in various DNA-repair processes upon DNA damage [17,21,22]. In view of these studies,

Abbreviations used: 9-1-1 complex, Rad9–Rad1–Hus1 complex; AP, apurinic/apyrimidinic; ATM, ataxia telangiectasia mutated; BER, base excision repair; DTT, dithiothreitol; Fen1, Flap endonuclease 1; GST, glutathione S-transferase; HAP1, human apurinic endonuclease 1; LP-BER, long-patch BER; PCNA, proliferating-cell nuclear antigen; pol, DNA polymerase; RF-C, replication factor C; SP-BER, short-patch BER; the prefix h denotes human.

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and of its localization on to DNA lesions, the 9-1-1 checkpoint complex could be a potential candidate for the role of 'recruiting platform' for DNA-repair effectors.

Recent investigations searched for a possible link between the human 9-1-1 complex and the BER pathway. First, we reported a physical and functional interaction of the 9-1-1 complex with the main BER pol, pol β [23]. Similar physical and functional interactions with the 9-1-1 complex were subsequently identified for human Fen1 [24,25] and for the MutY homologue of *Schizosaccharomyces pombe* [26]. In the present paper, we document the physical and functional interaction of another LP-BER component, DNA ligase I, with the 9-1-1 complex. We hypothesize that the alternative clamp 9-1-1 might be an important sensor and adapter for the BER machinery in human cells.

Table 1 The clamp PCNA and the alternative clamp and checkpoint sensor 9-1-1 complex can interact with components of the LP-BER machinery

LP-BER component	Interacting partner	Reference
DNA glycosylase	PCNA	[29]
MutY DNA glycosylase	9-1-1 complex	[26]
HAP1	PCNA	[30]
HAP1	9-1-1 complex	Not known
Pol β	PCNA	[31]
Pol β	9-1-1 complex	[23]
Fen1	PCNA	[32]
Fen1	9-1-1 complex	[24,25]
DNA ligase I	PCNA	[33]
DNA ligase I	9-1-1 complex	The present study

MATERIALS AND METHODS

Chemicals

[γ-^{32}P]dATP (3000 Ci/mmol) and unlabelled ATP were from Amersham Biosciences. DNA oligonucleotides were purchased from Microsynth GmbH (Balgach, Switzerland). DNase, RNase and protease-free formamide were from Acros Organics. T4 polynucleotide kinase was from New England Biolabs. All other reagents were from Merck, Fluka or Sigma.

Nucleic acid substrates

The sequences of oligonucleotides used to prepare the substrates for the DNA ligase I assays are the following: 46-mer, 5′-AGATTTTTCATTTGCTGCTGGCTCTCAGCGTGGCACTGTTGCAGGC-3′; 25-mer, 5′-CCTGCAACAGTGCCACGCTGAGAGC-3′; 19-mer, 5′-CAGCAGCAAATGAAAAATC-3′ (for schematic representations see Figure 2A). The 25-mer and 19-mer were labelled at the 5′-end in a buffer containing 70 mM Tris/HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT (dithiothreitol), an equimolar amount of [γ-^{32}P]ATP or unlabelled ATP and T4 polynucleotide kinase for 45 min at 37 °C. T4 polynucleotide kinase was heat-inactivated for 10 min at 80 °C, and free ATP was removed on Microspin™ G-25 columns. To generate the substrates for the DNA ligase I assays, the appropriate oligonucleotides were mixed in a 1:1 molar ratio in a buffer containing 50 mM Tris/HCl, pH 7.4 and 150 mM NaCl, heated for 10 min to 75 °C and slowly cooled to room temperature (20 °C).

Enzymes and proteins

Myoglobin was purchased from Serva Feinbiochemica (Heidelberg, Germany). hPCNA (human PCNA) was produced in *Escherichia coli* using the plasmid pT7/hPCNA and was purified to homogeneity as described in [27]. Human DNA ligase I was expressed in *E. coli* and was purified as described in detail by Jónsson et al. [28]. Untagged or histidine-tagged (His-tagged) 9-1-1 complexes were respectively obtained by co-expressing in Sf9 insect cells the three baculoviruses encoding the recombinant hRad1, hRad9 and hHus1 or recombinant hRad1, hRad9 and His-hHus1 (see Figure 2B). The 9-1-1 complex was subsequently purified as described in [23]. GST (glutathione S-transferase) was expressed in *E. coli* strain TG1 and was purified by binding on glutathione–Sepharose beads as described by Toueille et al. [23].

Pull-down assays

His-tagged human DNA ligase I bound to ProBond™ beads (Invitrogen) was incubated with purified 9-1-1 complex or purified GST as a negative control for 2 h at 4 °C in 50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 0.1 % (v/v) Nonidet P40, 1 mM 2-mercaptoethanol and 1 mM PMSF. After washing four times in the same buffer, the beads were heated for 5 min at 95 °C in Laemmli buffer, and the co-precipitated proteins were analysed by Western blot using the corresponding antibodies according to established methods.

Activity assays for DNA ligase I

The labelled DNA substrate presented in Figure 2(A) (25 fmol) was incubated for 20 min at 37 °C with recombinant human DNA ligase I in presence or absence of various amounts of the His-tagged 9-1-1 complex or PCNA, or myoglobin as a negative control. The reaction was performed in a final volume of 10 μl containing 40 mM Tris/HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP. After boiling for 5 min at 100 °C with formamide containing loading buffer, samples were electrophoresed through a 15 % denaturing polyacrylamide gel containing 7 M urea and 7 M formamide. Labelled oligonucleotides were detected by autoradiography and quantified by PhosphorImager analysis (Molecular Dynamics).

RESULTS AND DISCUSSION

The human checkpoint sensor and alternative DNA clamp 9-1-1 complex can interact with components of the LP-BER machinery

The clamp PCNA and the alternative checkpoint clamp, the 9-1-1 complex, can interact with components of the LP-BER machinery. It is known that PCNA, initially identified as a processivity clamp for pols δ and ε, can interact with more than 25 cellular proteins (reviewed in [11]). Among them there are components of the LP-BER machinery (Table 1). They include DNA glycosylase [29], HAP1 [30], pol β [31], Fen1 [32] and DNA ligase I [33]. We have initially shown that the 9-1-1 complex can interact physically with pol β *in vitro*, and functional analysis revealed that the 9-1-1 complex had a specific stimulatory effect on pol β activity [23]. Pol β stimulation resulted from an increase in its affinity for the primer template, and the interaction with the 9-1-1 complex stimulated deoxyribonucleotide misincorporation by pol β. Finally, the 9-1-1 complex enhanced DNA strand-displacement synthesis by pol β, an activity required for LP-BER, raising the possibility that the 9-1-1 complex might attract pol β to DNA-damage sites, thus connecting checkpoints and DNA repair directly. More recently, it was found that the 9-1-1 complex can interact with and stimulate Fen1 [24,25], and that the 9-1-1 complex of *S. pombe* can interact with DNA glycosylase MutY homologue [26].

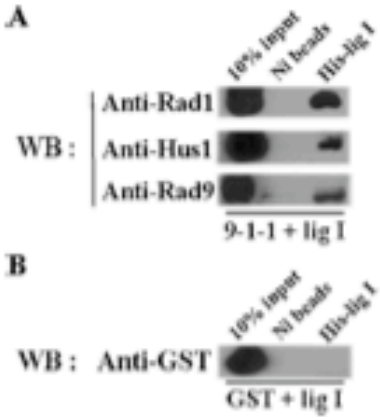

Figure 1 The human 9-1-1 complex interacts with human DNA ligase I

Pull-down of the 9-1-1 complex and DNA ligase I. (**A**) His pull-down experiments were performed in the presence of either His-tagged DNA ligase I (5 μg) or nickel beads alone, and purified 9-1-1 complex (3.6 μg). (**B**) The control His pull-down experiments were performed in the presence of either His-tagged DNA ligase I (5 μg) or nickel beads alone, and purified GST (3.6 μg). The presence of co-precipitated proteins was determined by SDS/PAGE followed by Western blot (WB) analysis. lig I, DNA ligase I.

The human 9-1-1 complex can physically interact with DNA ligase I

Based on these results, we first tested whether the 9-1-1 complex can interact with DNA ligase I. As shown in Figure 1, the 9-1-1 complex can interact physically with DNA ligase I in a His pull-down experiment performed after incubation of the purified 9-1-1 complex with His-tagged DNA ligase I or with nickel beads alone (Figure 1A). Western blot analysis against Rad9, Rad1 and Hus1 showed a specific co-precipitation of the complex with His-tagged DNA ligase I, but not with nickel beads used as a control. To check for the specificity of this interaction, GST was incubated under the same conditions with DNA ligase I or nickel beads. As shown in Figure 1(B), GST was not co-precipitated with either DNA ligase I or the nickel beads, confirming that the 9-1-1 complex interacts specifically with DNA ligase I *in vitro*.

The human 9-1-1 complex can stimulate DNA ligase I

Next, we tested the effect of the 9-1-1 complex on the activity of DNA ligase I *in vitro*. From Figures 2(C) and 2(D), it is evident that the 9-1-1 complex can stimulate DNA ligase I even at a 1:1 ratio (Figure 2C, compare lanes 3 and 4, and see quantification in Figure 2D). Stimulation of DNA ligase I activity up to 12-fold was observed at a 9-1-1 complex/DNA ligase I ratio of 27:1 (Figure 2C, compare lane 3 and 7, and see quantification in Figure 2D). The stimulation is specific, since adding the same amounts of myoglobin gave virtually no stimulation (Figure 2C, lanes 9–13, and see quantification in Figure 2D).

Human PCNA can stimulate human DNA ligase I slightly, but cannot prevent the stimulation of DNA ligase I by the human 9-1-1 complex

Since it is known that PCNA can interact with DNA ligase I [33], we next tested the effect of PCNA on DNA ligase I and compared it with the stimulatory effect of the 9-1-1 complex. Figure 3(A) (lanes 3–7) documents as a positive control the stimulation of DNA ligase I by the 9-1-1 complex. Furthermore, PCNA could, as expected, also stimulate DNA ligase I, but to a much lesser extent than the 9-1-1 complex (up to a 2.8-fold stimulation; Figure 3A, lanes 9–13 and Figure 3C-2). Next, we tested the

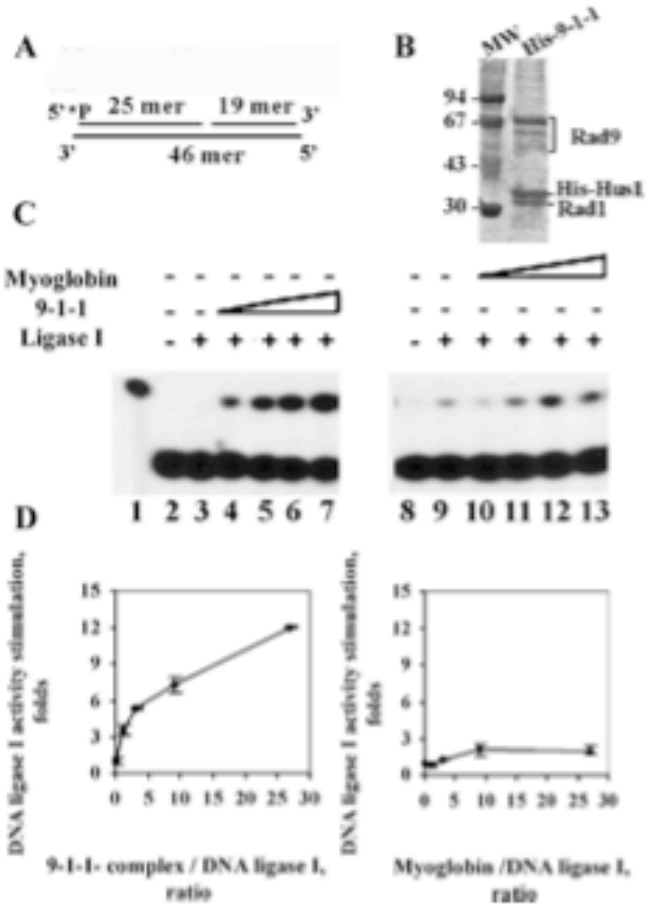

Figure 2 The human 9-1-1 complex can stimulate human DNA ligase I

DNA ligase activity was determined as described in the Materials and methods section. (**A**) Substrate used in DNA ligase assays. (**B**) SDS/PAGE, followed by Coomassie Blue staining of the purified His-9-1-1 complex used in the assays. MW, molecular-mass values. (**C**) Effect of the 9-1-1 complex on DNA ligase I activity. The assays were performed in the presence of 30 fmol of recombinant human DNA ligase I. Lane 1, 46-mer marker; lane 2, no enzyme control, 25-mer marker; lane 3, DNA ligase I alone; lanes 4–7, 30, 90, 270 or 810 fmol respectively of the purified recombinant human 9-1-1 complex was added; lane 8, no enzyme control; lane 9, DNA ligase I alone; lanes 10–13, 30, 90, 270 or 810 fmol respectively of myoglobin was added. (**D**) Quantification of the effect of the 9-1-1 complex (lanes 3–7 in **C**) and of myoglobin (lanes 9–13 in **C**) on DNA ligase I activity. The data represent the means for three experiments.

effect of PCNA in the presence of DNA ligase I and 9-1-1 complex at a ratio of 9:1 (Figure 3B, lanes 9–12). At this 9-1-1 complex/DNA ligase I ratio, stimulation of the ligase activity by the 9-1-1 complex was clearly visible, but not saturating, which allows detection of either a stimulatory or an inhibitory effect of PCNA. As expected, the 9-1-1 complex stimulated DNA ligase I (compare lanes 8 and 9 in Figure 3B), but upon further addition of PCNA, neither stimulation nor inhibition by PCNA was observed (Figure 3B, lanes 9–12, and Figure 3C-4). This suggests strongly that, once the 9-1-1 complex binds to DNA ligase I, PCNA is unable to interfere. Finally, we tested the effect of the 9-1-1 complex in the presence of a PCNA/DNA ligase I ratio of 9:1 (Figure 3B, lanes 3–6 and Figure 3C-3). The 9-1-1 complex could stimulate DNA ligase I even in the presence of excess PCNA, although the observed stimulation was slightly, but not significantly, weaker. These results suggest that, under the present conditions, PCNA does not prevent the stimulation by the 9-1-1 complex of DNA ligase I activity. Considering that the 9-1-1 complex is recruited on to DNA upon genotoxic stress, the stronger

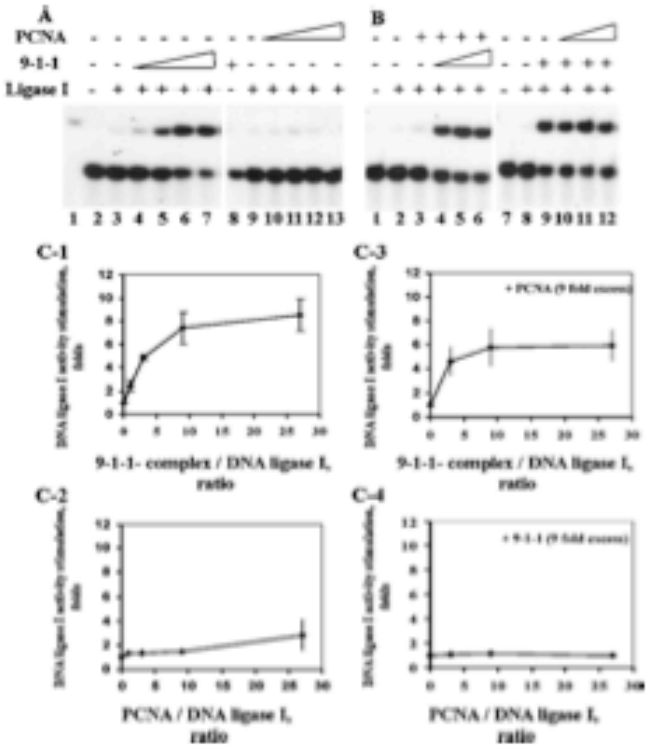

Figure 3 Human PCNA can slightly stimulate human DNA ligase I, but cannot prevent the stimulation of DNA ligase I by the human 9-1-1 complex

DNA ligase activity was determined as described in the Materials and methods section. (**A**) Effect of the 9-1-1 complex and PCNA on the activity of human DNA ligase I. The assays were performed in the presence of 30 fmol of recombinant human DNA ligase I. Lane 1, 45-mer marker; lane 2, no enzyme control, 25-mer marker; lane 3, DNA ligase I alone; lanes 4–7, 30, 90, 270 or 810 fmol respectively of the purified recombinant human 9-1-1 complex was added; lane 8, 2430 fmol of 9-1-1 complex was added in the absence of DNA ligase I, no ligase control; lane 9, DNA ligase I alone; lanes 10–13, 30, 90, 270 or 810 fmol of the purified recombinant human PCNA was added. (**B**) Effect of PCNA on the stimulation of DNA ligase I by the 9-1-1 complex. The assays were performed in the presence of 30 fmol of recombinant human DNA ligase I. Lane 1, no enzyme control, 25-mer marker; lane 2, DNA ligase I alone; lanes 3–6, DNA ligase in the presence of 270 fmol of PCNA and 0, 90, 270 or 810 fmol respectively of the 9-1-1 complex; lane 7, no enzyme control, 25-mer marker; lane 8, DNA ligase I alone; lanes 9–12, DNA ligase I and 270 fmol of 9-1-1 complex in the presence of 90, 270 or 810 fmol respectively of PCNA. (**C**) 1, Quantification of the effect of the 9-1-1 complex alone (lanes 3–7 in **A**); 2, quantification of the effect of PCNA alone (lanes 9–13 in **A**); 3, quantification of the stimulation by the 9-1-1 complex in the presence of PCNA (lanes 3–6 in **B**); 4, quantification of the PCNA effect in the presence of the 9-1-1 complex (lanes 9–12 in **B**). The data presented in (**C**) are the means for three experiments.

effect observed compared with PCNA may reflect the fact that the stimulation of DNA ligase I is greater, when its activity is needed as a priority at a place of DNA damage.

Conclusions

Most components of the LP-BER machinery can interact with the clamp PCNA. Moreover, as shown very recently by us and others, the checkpoint alternative clamp 9-1-1 complex can interact with and stimulate at least the four components, DNA glycosylase, pol β, Fen1 and DNA ligase I, of the LP-BER machinery. In this work we compared for the first time the effect of the 9-1-1 complex with that of PCNA on a LP-BER factor. We showed that the stimulation by the checkpoint sensor 9-1-1 complex is stronger than that by the classical DNA clamp PCNA. Moreover, the latter cannot prevent the stimulatory effect of the 9-1-1 complex on DNA ligase I. These findings now open the way to elucidate the *in vitro* and *in vivo* situations under which the clamp PCNA is 'replaced' by the alternative clamp 9-1-1. In our efforts to understand the role of the 9-1-1 complex in the process of BER, we have already characterized, in simple systems, the interaction of the 9-1-1 complex with different BER proteins, namely pol β [23], DNA ligase I (the present study) and Fen1 ([24,25], and E. Friedrich-Heineken, M. Toueille, B. Tännler and U. Hübscher, unpublished work). Hence, we are now in a position to establish a more physiological system including the respective clamp loaders RF-C for PCNA and its alternative clamp loader Rad17–RF-C_{2-5} for the 9-1-1 complex and a DNA template harbouring an AP site. This should allow us to test whether PCNA interferes with the effects of the 9-1-1 complex on different components and at different steps of the LP-BER pathway. Eventually, we will learn more about the possible alternative role of the two clamps in the function of the LP-BER mechanism.

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